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The Journal of Biochemistry, Vol. 44 No. 12 1957

STUDIES ON XANTHURENIC ACID

XIV. INHIBITORY ACTION OF 4-HYDROXY-8-METHOXY-QUINOLINE-2-CARBOXYLIC ACID, ETHEREAL SULFATE OF XANTHURENIC ACID AND KYNURENIC ACID ON THE DIABETOGENIC PROPERTY OF XANTHURENIC ACID

By YAHITO KOTAKE AND MURAJI KATO

(From the Biochemical Department, Wakayama Medical College, Wakayama)

(Received for publication, March 15, 1957)

In the previous report, xanthurenic acid, an abnormal metabolite of tryptophan, was pointed out by Kotake *et al.* (*1*) to be a diabetogenic substance. Oxine, likewise, was formerly reported by Okamoto (*2*) to be an agent diabetogenically active. On the basis of these two observations, it seems probable that the presence of free OH radical in the 8 position of quinoline ring is indispensable for diabetogenic activity of quinoline compound.

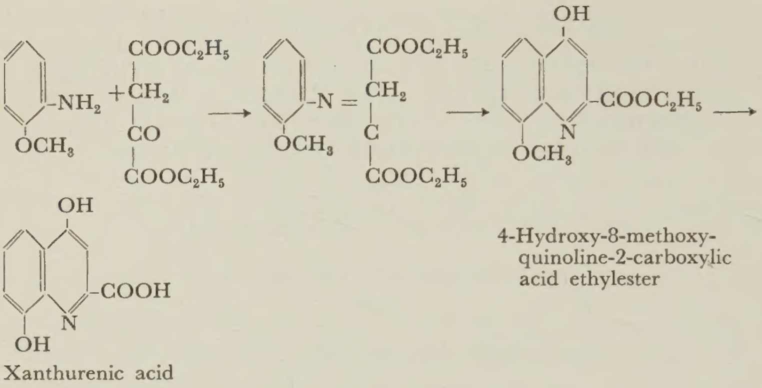
In this paper some additional evidences are presented showing that the quinoline compounds, deprived of free OH radical by methylation, as in 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid and by esterification with acid, as in 4,8-diethereal sulfate of xanthurenic acid exert not only no diabetogenic action by themselves but also somewhat antagonistic action toward xanthurenic acid. Further, it is interesting to note that kynurenic acid, which possesses no OH radical under consideration showed the quite similar behavior as those compounds above mentioned.

EXPERIMENTAL AND RESULTS

I. Experiment with 4-Hydroxy-8-methoxy-quinoline-2-carboxylic Acid—Preparation of pure xanthurenic acid and 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid ethyl ester were carried out as follows: These compounds were synthesized in accordance with the revised Musajo's method (*3*). In this case xanthurenic acid was obtained by demethylation of 4-hydroxy-8-methoxyquinoline-2-carboxylic acid ethyl ester (Diagram 1).

The preparation was often contaminated with trace of the acid

DIAGRAM I



form of the mother substance. This can be well recognized by means of paper chromatography. When the mixture of *n*-butanol, acetic acid and water (4:1:1) is used as a developer, the R_f value of xanthurenic acid is 0.48, while that of 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid 0.75. The corresponding spots can be confirmed by fluorescent test or color reactions. Namely the former gives pale blackish fluorescence under ultraviolet light, while the latter brilliant blue and the color reactions such as the Pauly's diazo, the Ehrlich's diazo and Fe^{+++} are positive with the former but all negative with the latter.

In the animal experiment, albino rats, fed more than a fortnight on a casein diet and weighing 200 g., were used. A group of the animals were intraperitoneally injected with 200 mg./kg. of xanthurenic acid, serving as a control. Another 3 groups were injected with 100 mg., 50 mg. or 20 mg./kg. of 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid ethyl ester respectively and 10 minutes later each reinjected intraperitoneally with 200 mg./kg. of xanthurenic acid. Then the blood sugar were determined by the King-Garner's method (*4*) with time. The results are illustrated in Figs. 1, 2 and 3.

It is now apparent that the change of blood sugar level such as the initial hyperglucemia followed by the hypoglucemia and then the second hyperglucemia caused by the injection of xanthurenic acid was completely suppressed by 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid ethyl ester even in such small dosis as one tenth of xanthurenic acid.

The same results were obtained by 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid, which is found rather as an impurity in the preparation of xanthurenic acid. (Fig. 4).

II. Experiment with Ethereal Sulfate of Xanthurenic Acid—Preparation of ethereal sulfate of xanthurenic acid (*5*) was carried out as follows:

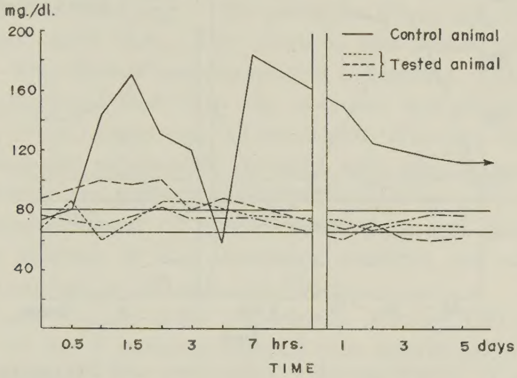


FIG. 1. Tested animals: xanthurenic acid 200 mg./kg. and 4-hydroxy-8-methoxy quinoline-2-carboxylic acid ethyl ester 100 mg./kg. injection

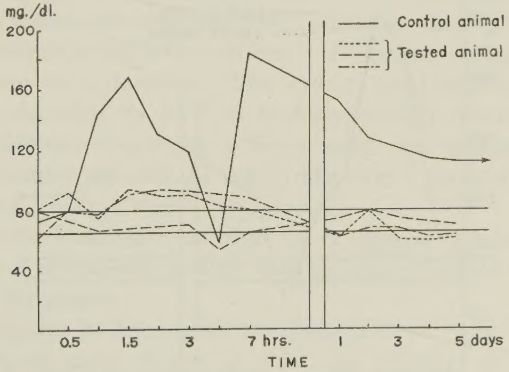


FIG. 2. Tested animals: xanthurenic acid 200 mg./kg. and 4-hydroxy-8-methoxy quinoline-2-carboxylic acid ethylester 40 mg./kg. injection.

A number of albino rats were injected with 20 mg. of xanthurenic acid (m.p. 296.5°). 24 hours urine was collected, filtered, adjusted to pH 3.0 and kept in an ice-box overnight. The whole urine was extracted with the mixture of ether and alcohol (15:1) and filtered through Amberlite IRA 410. Being concentrated, the filtrate was subjected to

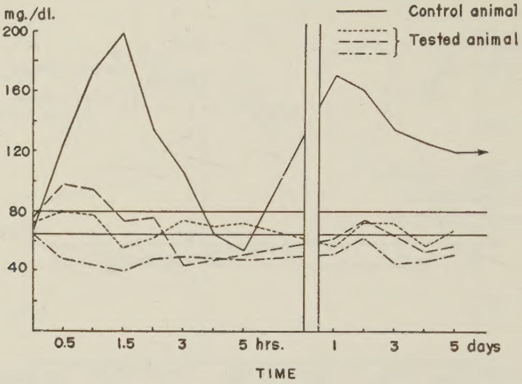


FIG. 3. Tested animals : xanthurenic acid 200 mg./kg. and 4-hydroxy-8-methoxy quinoline-2-carboxylic acid ethylester 20 mg./kg. injection.

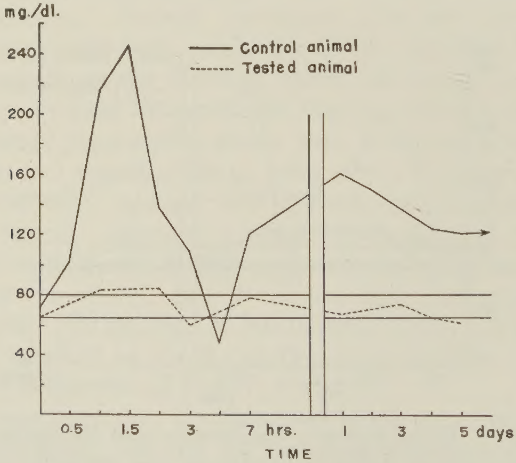


FIG. 4. Tested animals : xanthurenic acid 200 mg./kg. and 4-hydroxy-8-methoxy quinoline-2-carboxylic acid 50 mg./kg. injection.

paper chromatography using a mixture of methanol, *n*-butanol, benzene and water (2:1:1:1) as a developer. The dark purplish blue fluorescent spot (R_f 0.28) was cut out and its water extract was again paperchromatographed, using *n*-butanol: acetic acid: water (4:1:1) as a developer. The fluorescent spot (R_f 0.08) was again cut out and its extract was treated with a saturated mercury acetate solution, upon which a precipitate came out. The precipitate was suspended in water and decomposed with H_2S. The solution was mixed with methanol and filtered. The filtrate was evaporated to dryness. The residue was dissolved in methanol and then the solution was slowly evaporated, upon which a crystal came out. This crystal, although contained some amount of inorganic substances, revealed only one fluorescent spot on a paper chromatogram, corresponding to the ethereal sulfate of xanthurenic acid previously found by one of the authors and Nogami. Also the spectrum analysis of this substance confirms the above finding, showing two maxima at 246 mμ and 336 mμ.

But here still remains some uncertainty whether OH radical in the 4 position or in the 8 position entered into esterification with sulfulic acid or both of them.

It was found, however, that one molecule of sulfuric acid was separated by hydrolysis with 0.1 *N* HCl in sealed tube at 100° remaining a substance which showed paperchromatographycally R_f 0.14 and negative reactions toward any diazo color tests. Since this diazo reaction is assumed to be associated with free OH radical in the 8 position, it is almost certain that this radical is not free. Therefore, sulfuric acid should be detected from the 4 position. The authors tried once more to subject the substance showing R_f 0.14 to hydrolysis with more concentrated HCl (5 *N*). Herein one more sulfuric acid was separated and the remaining substance showed R_f 0.45 (developer: butanol, acetic acid and water) and positive diazo reactions.

It will be concluded after all that the crystalline compound obtained from the urine of albino rats treated with xanthurenic acid was diethereal sulfate of this substance.

The procedures in the animal experiment were quite the same as in the previous experiment except in this case 100 mg. or 75 mg./kg. of diethereal sulfate of xanthurenic acid was injected intraperitoneally.

The results of blood sugar determinations are illustrated in Figs. 5 and 6.

It will noticed that the initial hyperglucemia was not completely abolished but the second one was completely suppressed.

III. Experiment with Kynurenic Acid—Kynurenic acid here used was a pure one synthesized by prof. Sakan. The experimental condition was the same as the foregoing one except in this case either 100 mg. or 200 mg./kg. of kynurenic acid were injected.

The behavior of blood sugar is well illustrated in Figs. 7 and 8. It will be noticed that the administration of 100 mg. of kynurenic acid

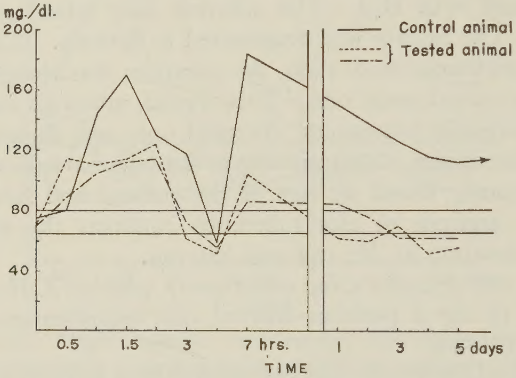


FIG. 5. Tested animals : xanthurenic acid 200 mg./kg. and ethereal sulfate of xanthurenic acid 75 mg./kg. injection.

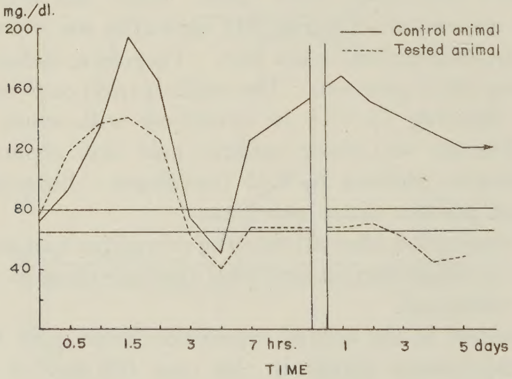


FIG. 6. Tested animals : xanthurenic acid 200 mg./kg. and ethereal sulfate of xanthurenic acid 75 mg./kg. injection.

showed no appreciable effect on the initial hyperglucemia due to xanthurenic acid, but a significant reduction of the second hyperglucemia.

In the case of 200 mg. of kynurenic acid, the suppressing effect are fairly apparent toward both hyperglucemia.

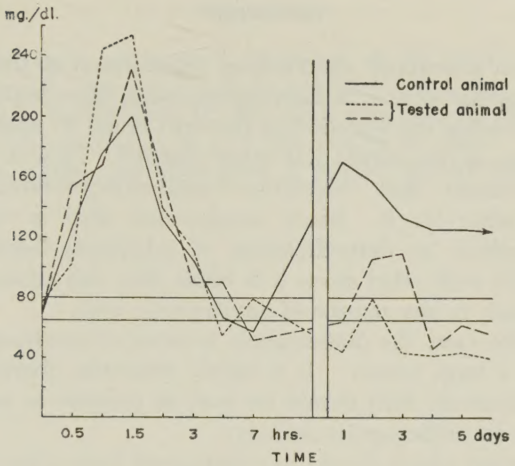


FIG. 7. Tested animals : xanthurenic acid 200 mg./kg. injection and kynurenic acid 100 mg./kg. injection.

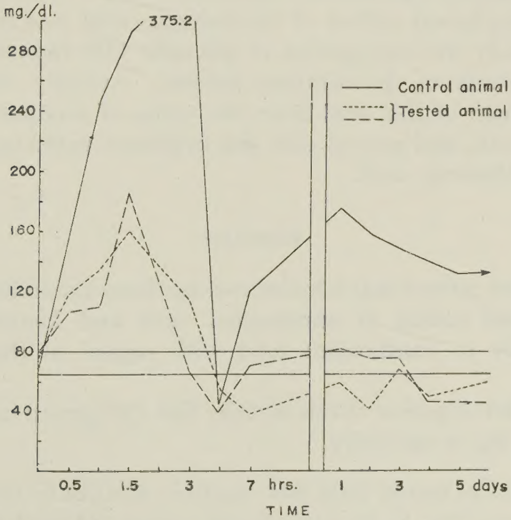


FIG. 8. Tested animals : xanthurenic acid 200 mg./kg. and kynurenic acid 200 mg./kg. injection.

DISCUSSION

It has been sometimes observed by the authors in the experiments on the diabetogenic action of xanthurenic acid, that hyperglucemia in albino rats following the injection of the acid failed to appear. At that time, the reason of this failure was quite obscure. Now it became clear by our experiment that 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid is responsible for it. Since xanthurenic acid is obtained after Musajo's method by demethylation of 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid ethyl ester, it is liable that this substance remains as a contaminant in the sample of xanthurenic acid.

If this is the case, the diabetogenic activity of xanthurenic acid will be reduced to a large extent. It is highly desirable, therefore, that the sample of xanthurenic acid should be pure as possible as we can for the use to estimate its diabetogenic activity.

Another point which should be emphasized here is the fact that OH in the 8 position of quinoline ring should be present and also free for the development of diabetogenic activity. Kynurenic acid, which corresponds to 8-dehydroxy xanthurenic acid, not only has no such activity but also acts as inhibitory to xanthurenic acid though in less degree than 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid. As another example in this line diethereal sulfate of xanthurenic acid was considered. In the animal body the conjugation of phenolic OH radical with sulfuric acid is the common detoxicating process. Actually we obtained a diethereal sulfate of this acid from the urine of animals injected with xanthurenic acid, and proved that this substance acted quite antagonistically to xanthurenic acid.

SUMMARY

It has been proved that 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid, diethereal sulfate of xanthurenic acid and kynurenic acid act antagonistically to xanthurenic acid with regard to the diabetogenic activity.

For the development of this activity free OH group in the 8-position of quinoline ring is necessary.

In conclusion, we heartily thank Prof. Yashiro Kotake for his pertinent advice and encouragement, Prof. K. Kodama for his revision, and also heartily thank Prof. T. Sakan for his advice to synthesis of the compounds necessary for this experiment.

REFERENCES

(*1*) Kotake, Yahito, and Inada, T., *J. Biochem.*, **40**, 291 (1953)
(*2*) Okamoto, K., *Acta scholae medicales universitatis in Kioto*, **27**, 43 (1949)
(*3*) Musajo, L. and Minchili, M., *Ber. deutsch. Chem. Ges.*, **74**, 1839 (1941)
(*4*) King, E. J. and Garner, R. J., *J. Clin. Path.*, **1**, 30 (1947)
(*5*) Kotake, Yahito, and Nogami K., *J. Biochem.*, **41**, 621 (1954)

The Journal of Biochemistry, Vol. 44, No. 12, 1957

PHYSICO-CHEMICAL STUDIES ON TAKA-AMYLASE A

II. ON THE COMPACTNESS AND THE HYDRATION OF THE MOLECULE

By TOSHIZO ISEMURA AND SHIZUE FUJITA

(From the Laboratory of Colloid Science, the Institute of Scientific and Industrial Research, Osaka University, Sakai, Osaka)

(Received for publication, June 6, 1957)

We have reported in the previous paper (*1*) the results of size and shape determination of Taka-Amylase A by the measurements of ultracentrifugal sedimentation, diffusion, and viscosity. The molecular weight of this protein was found concordantly to be 51,000 from three different combinations of measurements, namely, sedimentation and diffusion, sedimentation and viscosity, and diffusion and viscosity according to Simha-Perrin's treatment (*2*), if we assume that the Taka-amylase A molecule is of the prolate ellipsoid and scarcely hydrated.

Contrary to the treatment of Simha-Perrin which assumes the molecule to be rigid, Scheraga and Mandelkern (*3*) presented a new theory assuming that the solvent flows through the domain of the protein molecule. They defined the hydrodynamically effective volume in which the effect of molecular flexibility, and the effect of hydration were included without specifying the amount of hydration. The hydrodynamically effective volume calculated by their theory would be in-accord with the anhydrous molecular volume only when the protein molecule is rigid and anhydrous. In this point of view the hydrodynamically effective volume of Taka-amylase A was calculated and found that this is nearly the case. On the other hand, it was noticed that the observed partial specific volume of this protein is considerably less than the calculated value by the Cohn-Edsall's procedure (*4*) of summation of the specific volumes of constituent amino acids. The small partial specific volume of Taka-amylase A suggests a compact and rigid structure which may be attributed to the occupation of interstices between the polypeptide helices in the molecule by the prosthetic groups.

EXPERIMENTAL

The details of the experiments were described in the previous paper (*1*).

RESULTS AND DISCUSSION

As has been shown in Table VI in the previous paper (*1*), the values of molecular weight of Taka-amylase A estimated by three different procedures are in good agreement with each other if we assume that the molecule is of the prolate ellipsoid in shape and scarcely hydrated. The frictional ratio of the molecule and the length of minor axis calculated on this assumption are also in accord with the experimental findings. However, it is very difficult to accept that the protein molecules exists in nearly anhydrous state in solution. Nevertheless, if the Simha-Perrin procedure used previously is correct, we are compelled to conceive that the Taka-amylase A molecule may be in such a state under our experimental condition, *i.e.*, in 0.2 M acetate buffer at pH 4.8.

Our conclusion that the Taka-amylase A molecule might be poorly hydrated has been reached by the Simha-Perrin's treatment assuming that the protein molecule may be of rigid prolate ellipsoid. According to their treatment, actual volume of protein molecule in solution will be expressed as $M\bar{v}/N+Mw/N\rho$, which is assumed to correspond to the hydrodynamically effective volume, where M is the anhydrous molecular weight of the protein, $\bar{v}$ the partial specific volume of the anhydrous protein, ρ the density of the solvent, w the weight of solvent combining with a gram of the anhydrous protein. Then, $M\bar{v}/N$ is the contribution of anhydrous protein and $Mw/N\rho$ the volume increment by hydration. In this treatment, all the following effects were neglected, namely, possible flow of solvent through the domain, deviation of the shape of the domain from that of an ellipsoid of revolution, deformation of the domain by the hydrodynamic forces, selective adsorption from mixed solvent, electrostriction, and similar effects. Scheraga and Mandelkern thought that it is indispensable to take these effects into consideration.

According to them, the shape factor β which is the function of the axial ratio of effective hydrodynamic ellipsoid was calculated as follows,

$$\beta=\frac{Ns[\eta]^{1/3}\eta}{M^{2/3}(1-\bar{v}\rho)} \qquad (1)$$

$$\beta=\frac{D[\eta]^{1/3}M^{1/3}\eta}{kT} \qquad (2)$$

Substitution of the values of the sedimentation constant, $s=4.67$ svedberg, the diffusion coefficient, $D=7.37\times10^{-7}$ cm^2/sec., the intrinsic viscosity, $[\eta]=0.034$ dl./g., and the partial specific volume, $\bar{v}=0.700$ ml./g. for

Taka-amylase A determined previously (*1*) into Equation (1) or (2) led to the β value of 2.22×10^6 or 2.21×10^6, respectively. Since β is larger than 2.15×10^6, the effective hydrodynamic ellipsoid may be of prolate ellipsoid. The axial ratio, a/b, was estimated to be 4.7 or 4.3 respectively from these data. The hydrodynamically effective volume V_e of Taka-amylase A molecule corresponding to the β value estimated from Equation (1) or (2) was calculated, using $s = M(1 - \bar{v}\rho)/Nf$ or $D = kT/f$. It was found to be 5.32×10^{-20} or 5.74×10^{-20} ml., respectively. On the other hand, the effective volume of Taka-amylase A calculated by $M\bar{v}/N$ assuming the molecule to be rigid and anhydrous is 5.93×10^{-20} ml. This corresponds nearly to the hydrodynamically effective volume calculated by Scheraga-Mandelkern's treatment just mentioned. Comparison of the Simha-Perrin's treatment with that of Scheraga-Mandelkern for Taka-amylase A is given in Table I.

TABLE I

Comparison of the Simha-Perrin Treatment with That of Scheraga-Mandelkern for the Data of Taka-amylase A

Scheraga-Mandelkern			Simha-Perrin	
	from Eq. (1)	from Eq. (2)		
$\beta \times 10^{-6}$	2.22	2.21		
a/b (prolate)	4.67	4.33	*a/b* (prolate, anhydrous)	4.17
$V_e \times 10^{20}$ *ml.*	5.32	5.74	$M\bar{v}/N \times 10^{20}$ *ml.*	5.93

The effective volumes calculated by Scheraga-Mandelkern's procedure and $M\bar{v}/N$ values of some proteins are listed in Table II as well as the molecular weights, intrinsic viscosities, sedimentation constants and partial specific volumes used for calculation. For example, V_e value of a ichthyocol parent gelatin is 5.09×10^{-20} ml. and far less than the volume of the anhydrous protein, namely, of 8.16×10^{-20} ml. (*5*). In order to keep these volumes in harmony with each other, we must attribute negative hydration to the gelatin molecule. This contradiction may be caused by the fact that the gelatin molecule is of partially free drainage, and that the Simha-Perrin's treatment assuming a rigid ellipsoidal model would not be hold for this protein. Similar relations were found with horse serum albumin and egg albumin. On the other hand, V_e is nearly equal to, even slightly larger than $M\bar{v}/N$ with tobacco mosaic virus of which amount of hydration is considered to be small

(*6, 7*) and to be rather compact molecule (*8*).

The amount of hydration for tabacco mosaic virus has been estimated by Schachaman and Lauffer (*7*) as 0.15 ml./ml. virus. This estimation is open to some question, but it seems highly probable that the hydration is very likely less than for most smaller proteins.

From the facts mentioned above, it is probable that the Taka-amylase A molecule might be of the relatively rigid and scarcely hydrated.

Recently Polson (*9*) showed that the following relation can be applied to most proteins in aqueous solution,

$$MD^3[\eta]=k \qquad (3)$$

where k appears to be sensibly constant, which is 8.06×10^{-16}. Substituting the values of M, D and $[\eta]$ for Taka-amylase A into Equation (3), the k value is found to be 6.94×10^{-16}. This smaller value of k might be attributed to the high degree of compactness and the scarce hydration of this protein, although the unambiguous explanation cannot be offered at present.

TABLE II

Comparison of the Hydrodynamically Effective Volumes, V_e, calculated by Scheraga-Mandelkern's Treatment with the Anhydrous Molecular Volumes, $M\bar{v}/N$, of Some Other Proteins

Protein	$V_e\times 10^{20}$	$M\bar{v}/N \times 10^{20}$	Data used for calculation			
			$[\eta]$	$s\times 10^{13}$	$\bar{v}$	M
Gelatin	5.09(1)	8.16(1)	0.34(1)	0.34(1)	0.705(1)	69,700(1)
Egg albumin	2.40	5.47	0.043(2)	3.55(3)	0.749(4)	44,000(5)
Horse serum albumin	6.38	8.69	0.049(2)	4.46(6)	0.748(7)	70,000(6)
β-Lactoglobulin	4.54	5.18	0.045(2)	3.12(8)	0.751(8)	41,500(8)
Tobacco mosaic virus	4313	4007	0.285(2)	185(9)	0.727(9)	33,200,000(9)

References:

(*1*) Gallop, P.M., *Arch. Biochem. Biophys.* **54**, 501 (1955)
(*2*) Polson, P., *Kolloid-Z.*, **38**, 51 (1939)
(*3*) Mean value obtained by a number of different observers in Uppsala.
(*4*) Svedverg, T., and Sjögren, B., *J. Am. Chem. Soc.*, **50**, 525 (1928)
(*5*) Lamm. O., and Polson, A., *Biochem. J.* **30**, 528 (1936)
(*6*) Kekwick, R. A., *Biochem. J.*, **32**, 552 (1938)
(*7*) Svedberg, T., and Sjögren, B., *J. Am. Chem. Soc.*, **50**, 3318 (1928)
(*8*) Pedersen, K. O., *Biochem. J.*, **30**, 961 (1936)
(*9*) Lauffer, M. A., *J. Am. Chem. Soc.*, **66**, 1188 (1944)

It is known (*10*) that the observed partial specific volumes obtained by density measurements are in good agreement with those calculated by the following equation (*4*) with many proteins as shown in Table III.

$$\bar{v}=\sum v_i w_i/\sum w_i \qquad (4)$$

TABLE III*

Partial Secific Volumes of Proteins

Protein	Specific volume observed (*ml/g.*)	Specific volume calcd. from amino acid composition (*ml/g.*)
Silk fibroin (suspended in H_2O)	0.701	0.689
Ribonuclease	.709	.703
Wool (suspended in H_2O)	.716	.712
Lysozyme	.722	.717
Fibrinogen (human)	.725	.723
α-Casein	.728	.725
Chymotrypsinogen	.73	.734
Serum albumin (bovine)	.734	.734
Casein (unfractionated)	.731	.731
Insulin (Zn)	.735	.724
D-glyceraldehyde phosphate dehydrogenase	.737	.743
Aldolase	.740	.743
β-Casein	.741	.743
Ovalbumin	.745	.738
Hemoglobin (horse)	.749	.741
β-Lactoglobulin	.751	.746
Butulinus toxin	.75	.736
Gelatin	.682	.707
Edestin	.744	.719

* taken from McMeekin, T. L., and Marshall, K., *Science*, **116**, 142 (1952)

where $\bar{v}$ is the partial specific volume of the protein, v_i the specific volume and w_i its weight percent of the i-th species of amino acid residue in the protein. This agreement suggests that the effect of electrostriction is compensated by another factor. It seems most likely that this is a steric effect, which makes a small volume, an unoccupied space, inaccessible to solvent, due to the manner of the folding and packing of the peptide

TABLE IV

Partial Specific Volume of Taka-amylase A calculated from the Specific Volumes of Constituent Amino Acid and Carbohydrate Residues

	Percent by weight⁽¹⁾ of amino acid and carbohydrate residue w_i	Specific volume of⁽²⁾ amino acid and carbohydrate v_i	$v_i w_i$
Leucine	7.17	0.90	6.45
Isoleucine	4.49	.90	4.04
Phenylalanine	3.78	.77	2.91
Tyrosine	8.60	.71	6.11
Valine	3.97	.86	3.41
Methionine	1.94	.75	1.46
Alanine	5.43	.74	4.02
Glutamic acid	6.10	.66	4.03
Threonine	9.08	.70	6.36
Aspartic acid	4.18	.60	2.51
Asparagine	10.08	.62	6.25
Serine	5.37	.63	3.38
Glycine	5.01	.64	3.21
Arginine	2.45	.70	1.72
Lysine	5.19	.82	4.26
Hystidine	1.78	.67	1.19
Cystine (+cystein)	1.37	.63	0.86
Tryptophan	3.60	.74	2.66
Proline	3.52	.76	2.68
Total	93.11		67.51
Mannose	2.45 (8 moles)	.66	1.62
Xylose	0.25 (1 mole)	.67	0.17
Hexosamine	0.61 (2 moles)	.68	0.41
Total	96.42		69.71

Partial specific volume $\bar{v}=\sum v_i w_i / \sum w_i = 69.71/96.42 = 0.723$ ml./g.

If it is assumed that the carbohydrates do not contribute to the molecular volume but only to the weight,

$\bar{v}=67.51/96.42=0.700$ ml./g.

(1) The data of the amino acid composition of Taka-amylase A is taken from Akabori, S., Ikenaka, T., Hanafusa, H., and Okada, Y., *J.*

chains (*11*).

The partial specific volume of Taka-amylase A was found experimentally to be 0.700 ml./g. (*1*), whereas the volume calculated by Equation (4) was 0.723 ml./g. as shown in Table IV. The difference between the observed and the calculated values is fairly marked and beyond the experimental error.

Taka-amylase A contains carbohydrates such as 8 moles of mannose, 1 mole of xylose and 2 moles of hexosamine as prosthetic groups (*12*). If these sugars occupy the interstices between polypeptide helices in the protein molecule as in the case of heme in cytochrome c molecule (*13*), they contribute only to the weight of the protein but not to the total volume. Accordingly, the partial specific volume may be calculated by dividing the sum of the volumes of the constituent amino acid residues, $\Sigma v_i w_i$, by the total weight including the prosthetic groups, Σw_i. The calculated value of the partial specific volume is 0.700 ml./g. and coincides completely with the experimental value. It seems likely that the occupation of the interstices by sugar molecules suppresses the accessibility of hydration sites inside the molecule to water and the swelling of the protein molecule.

Although hydroxyl groups of sugar molecules cannot occupy all of the hydration sites of the interstices between polypeptide helices of the molecule since the content of sugars is only three percent in weight, the inference such as mentioned above matches with the view that Taka-amylase A has a compact structure and is scarcely hydrated.

With egg albumin which also contains sugar as a prosthetic group, both the observed and the calculated partial specific volumes are nearly equal to each other, as shown in Table III. According to Porter (*14*), sugar combines at the amino end of the polypeptide chain in this protein. It may exist outside the molecule and con-

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Biochem., **41**, 803 (1954) and that of the carbohydrate components from Hanafusa, H., Ikenaka, T., and Akabori, S., *J. Biochem.*, **42**, 55 (1955).

(2) The specific volume values of the amino acid residues are the values given by Cohn, E. J., and Edsall, J. T., *Proteins, Amino Acids and Peptides*, Reinhold, New York, pp. 370 (1943) excepting that of cystine. 0.63 was used for the volume of cystine residue (McMeekin, T. L., Groves, M. L., and Hipp, N. J., *J. Am. Chem. Soc.*, **71**, 3298 (1949)) instead of 0.61, as given in Cohn and Edsall.

The specific volume values of the carbohydrate residues were calculated as the sum of the volume increments for the constituent atoms and groups (Cohn, E. J., and Edsall, J. T., *Proteins, Amino Acids and Peptides*, Reinhold, New York, pp. 157 (1943)).

tribute to both the volume and the weight of the protein.

Generally, it has been vaguely accepted that the hydration of protein molecule occurs on the surface of globular protein molecules. It is supposed, however, that some of the hydration sites are in intramolecular interstices. When the molecule is rigid and compact, and some of the interstices are occupied by prosthetic groups, the hydration may be suppressed considerably. This may be the case of Taka-amylase A molecule.

SUMMARY

1. The hydrodynamically effective volume, V_e, of Taka-amylase A was calculated according to the treatment of Scheraga and Mandelkern. It was found to be nearly equal to $M\bar{v}/N$, the anhydrous volume of the protein.

2. The hydrodynamically effective volume, V_e, of some other proteins were also calculated and compared with $M\bar{v}/N$ values of the respective proteins. V_e is far less than $M\bar{v}/N$ with flexible and hydrated protein, whereas both volumes are nearly equal with rigid anhydrous protein.

3. The observed partial specific volume by density measurement of Taka-amylase A is 0.700 ml./g. and markedly less than the calculated value of 0.723 ml./g. by the Cohn-Edsall method. The deviation may be caused by the occurrence of prosthetic sugar groups in the interstices between polypeptide helices in the molecule without any contribution to the molecular volume.

These facts mentioned above support the view that Taka-amylase A molecule may be rigid and compact and scarcely hydrated.

In conclusion, the authors express their hearty thanks to Prof. S. Akabori and Mr. T. Ikenaka for supplying the valuable sample. The authors are also indebted to the Ministry of Education for a grant.

REFERENCES

(*1*) Isemura, T., and Fujita, S., *J. Biochem.*, **44**, 443 (1957)
(*2*) Simha, R., *J. Phys. Chem.*, **44**, 25 (1940)
Perrin, F., *J. phys. radium*, [7] **7**, 1 (1936)
Mehl, J. W., Oncley, J. L., and Simha, R., *Science*, **92**, 132 (1940)
Oncley, J. L., *Ann. New York Acad. Sci.*, **41**, 121 (1941)
(*3*) Scheraga, H. A., and Mandelkern, L., *J. Am. Chem. Soc.*, **75**, 179 (1953)
(*4*) Cohn, E. J., and Edsall, J. T., Proteins, Amino Acids and Peptides, Reinhold,

New York, pp. 370 (1943)
(*5*) Gallop, P. M., *Arch. Biochem. Biophys.*, **54**, 501 (1955)
(*6*) Lauffer, M. A., *J. Am. Chem. Soc.*, **66**, 1188 (1944)
(*7*) Schachman, H. K., and Lauffer, M. A., *J. Am. Chem. Soc.*, **71**, 536 (1949)
(*8*) Bernal, J. D., and Fankuchen, I., *J. Gen. Physiol.*, **25**, 111, 147 (1941)
(*9*) Polson, A., *Biochem. Biophys. Acta*, **21**, 185 (1956)
(*10*) McMeekin, T. L., and Marshall, K., *Science*, **116**, 142 (1952)
Edsall, J. T., The Proteins, ed. by Neurath, H., and Bailey, K., Academic Press Inc., New York, **Vol. IB**, pp. 562 (1953)
(*11*) Charlwood, P. A., *J. Am. Chem. Soc.*, **79**, 776 (1957)
(*12*) Hanafusa, H., Ikenaka, T., and Akabori, S., *J. Biochem.*, **42**, 55 (1955)
(*13*) Ehrenberg, A., and Theorell, H., *Acta Chem. Scand.*, **9**, 1193 (1955)
(*14*) Porter, R. R., *Biochem. J.*, **46**, 473 (1950)

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PROLINE OXIDIZING ENZYME SYSTEM IN A HALOPHILIC BACTERIUM No. 101

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(Received for publication, June 11, 1957)

Few reports are available on the enzymochemical studies of the halotolerant and halophilic bacteria, although they represent a most interesting ecological group of organisms and are of some importance in food industries (*1*, *2*). In a series of studies on the enzyme systems responsible for the glutamic acid metabolism in these bacteria, the author previously reported on the transaminase system in a halophilic bacterium No. 101 isolated from salt whales (*3*, *4*). This paper describes some results obtained in regard to the oxidative enzyme systems, especially the metabolic pathways of proline, in this bacterium.

METHODS

Preparation of Cell Suspension--Halophilic *Pseudomonas* strain No. 101 was grown on a shaker at 30° in 500 ml. flasks containing 100 ml. of medium with the following composition : Ajinomoto's " Mieki " (solution of amino acid mixture prepared from HCl-hydrolysate of soybean meal, 20 g. of nitrogen per liter), 10 per cent ; NaCl, 10 per cent ; pH 7.2. After 48 hours the cells were harvested by centrifugation, washed twice with 10 per cent saline, and suspended in 10 per cent saline.

Test for Oxidizing Activity—Reaction mixtures for testing oxidizing activity usually contained 0.2–0.3 ml. of the cell suspension, 10 μM of substrate and 1 mM of NaCl in a final volume of 1 ml. of 0.1 *M* phosphate or veronal buffer, pH 7.0. The uptake of oxygen was determined manometrically at 30°.

Determination of Proline—Proline was determined by the photometric method of F. P. Chinard (*5*). After removing the cells by centrifugation or addition of 0.1 volume of 25 per cent trichloracetic acid, 1 ml. of the reaction mixture to be analyzed was mixed with 1 ml. of glacial acetic acid and 1 ml. of the reagent solution (25 mg. of ninhydrin dissolved in 1 ml. of the mixture of 6 *M* phosphaoric cid and glacial acetic acid (4:6)). After heating at 100° for 60 minutes followed by cooling to room temperature, the reaction mixture was filled up to 5 ml. with glacial acetic acid. The optical densities were estimated at 515 mμ.

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Determination of Glutamic γ-Semialdehyde—To 1 ml. of the solution to be analyzed, which contained 1–10 μM of glutamic γ-semialdehyde, were added 1 ml. of 0.1 *M* phosphate buffer pH 7.0 and 1 ml. of 0.015 *M* *o*-aminobenzaldehyde (synthesized from *o*-nitrobenzaldehyde (*6*), m.p. 37–8°). After filling up to 5 ml. with water and standing for 10 minutes, the optical densities were estimated at 435 mμ against the reagent blank (Fig. 1). The standard curve was fairly a straight line.

Determination of Glutamic Acid—Glutamic acid was determined manometrically by decarboxylation with specific L-glutamic decarboxylase of *E. coli* Crookes strain (*4*).

Determination of Bisulfite Binding Compounds—Bisulfite binding compounds were determined iodometrically by the method of Clift and Cook (*7*).

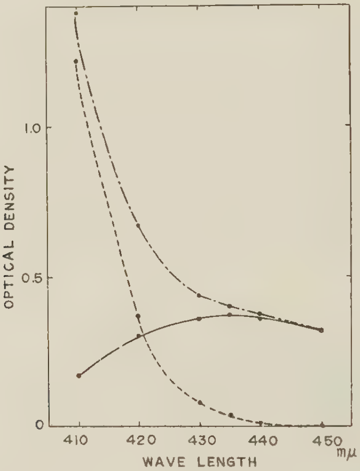


FIG. 1. Absorption curve of glutamic γ-semialdehyde-*o*-aminobenzaldehyde color reaction at pH 7.0.
–•–•– glutamic γ-semialdehyde+*o*-aminobenzaldehyde ; - - - - *o*-aminobenzaldehyde ; —— difference between above two curves.

Paper Chromatographic and Autoradiographic Detection of the Proline Metabolites—After removing the cells by centrifugation and acidifying with 3 *N* HCl, reaction mixtures were dried up in a vacuum desiccator and then extracted with warm 95 per cent ethanol. This ethanol extracts were dried up *in vacuo*, dissolved in a small quantity of water, and spotted on a Toyo No. 51 filterpaper sheet to be developed by a mixture of either *n*-butanol-acetic acid-water (4 : 1 : 1) or phenol-water (10 : 2 *v*/*v*) in the presence of 0.1 per cent NH_3. The chromatograms were detected by spraying 0.1 per cent ninhydrin in butanol saturated with water. For the detection of radioisotopic substances, the autoradiograms were obtained by contacting the paper with X-ray film for 2–4 days.

Isotopic Proline—The uniformly labeled C^{14}-L-proline was used in this study which was separated by ion exchange chromatography from the protein hydrolysate of *Chlorella ellipsoidae* cultivated with $C^{14}O_2$ in the author's laboratory (*8*, *9*). The purity of this preparation was confirmed by paper chromatography.

DL-*Glutamic γ-Semialdehyde*—DL-Glutamic γ-semialdehyde was obtained from its dimethyl acetal (217–8° decomp.) by heating the solution in 0.2 *N* acetic acid for a few minutes.

RESULTS

Effect of pH—The pH-activity curve for the oxidation of proline was determined for comparison with that of glutamic acid. As shown in Fig. 2, the optimum pH was 7.5 and 8.0 for the oxidation of proline and glutamic acid respectively.

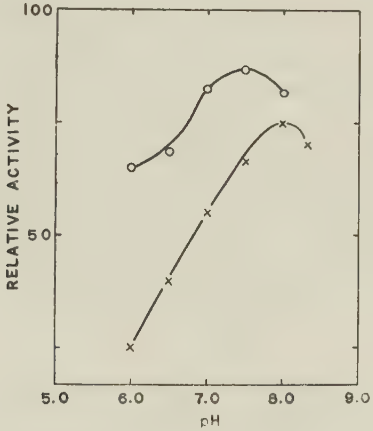


FIG. 2. Effect of pH on enzymatic activity of cell suspension for the oxidation of proline and glutamic acid. Reaction mixture contained 30 μN of L-proline (-O-O-) or L-glutamic acid (−×−×−) and 0.2 ml. of cell suspension and 6 per cent NaCl in a final volume of 3.0 ml. of 0.1 *M* phosphate buffer at pH as given in the figure. Oxygen uptake was observed manometrically at 30°.

Effect of NaCl Concentration—The sodium chloride concentration-activity curves were also determined for the oxidation of proline and glutamic acid (Fig. 3). The activity was varied more largely with the NaCl concentration for the oxidation of proline than for that of glutamic acid, and showed clearly a optimum concentration around 5.5 per cent (*w/w*).

Effect of Age of the Cultures—When cells were removed from growing a culture of this bacterium and tested for enzymic activity for the oxidation of proline and glutamic acid, both activities and the activity ratio varied with the age of the culture as shown in Table I. If peptone-meat extract was used instead of the " Mieki " as culture medium (*3*), the cells showed lower activities for the oxidation of proline and glutamic acid after 30 hours' growth.

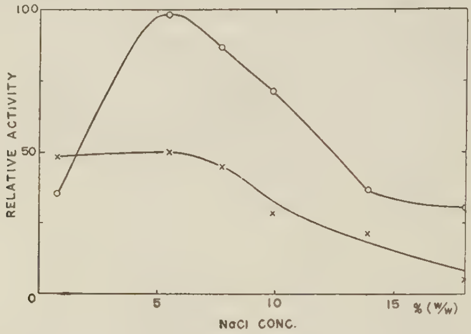


Fig. 3. Effect of NaCl concentration on enzymatic activity of cell suspension for the oxidation of proline and glutamic acid. Reaction mixture contained 30 μM of L-proline (-O-O-) or L-glutamic acid (-×-×-) and 0.2 ml. of cell suspension and NaCl as given in the figure in a final volume of 3.0 ml of 0.1 *M* phosphate buffer pH 7.0. Oxygen uptake was observed manometrically at 30°.

Table I

Effect of Age of the Cultures on the Oxidizing Activity of the Cells for Proline and Glutamic Acid

Growth time	Cell concn. in culture medium	Oxidizing activity Q_{O_2} (N)		
		L-Proline	L-Glutamate	Ratio
(*hrs.*)				
24	700	59.2	42.0	0.71
30	4,600	52.6	31.4	0.60
38	6,550	39.1	28.6	0.73
46	7,100	38.6	37.3	0.97

Inhibitors—It has been shown usually that proline is metabolized *via* glutamic acid *in vivo*. When proline is oxidized under the conditions which cause the suppress of the oxidation of glutamate, the accumulation of glutamic acid would cocur. From this consideration the inhibition effect of various compounds were examined as shown in Table II. There are some reports indicating that $FeCl_2$, $MgSO_4$, $CuSO_4$ and Pb acetate exert no effect on glutamic dehydrogenase at the concentration of less than 2×10^{-4} *M* per litre, Cu^{++} inhibits at 10^{-5} *M* per litre, and Co^{++} and Zn^{++} have no effect for proline oxidase (*10, 11*). From Table II, $NaAsO_2$, $FeCl_3$, $ZnSO_4$ and $CoSO_4$ inhibit more strongly the glutamic acid oxidation than the proline oxidation.

Table II

Effect of Various Substances Inhibitory to the Oxidation of Proline and Glutamic Acid

Inhibitor	Concn. $\times 10^{-4}$ *M/litre*	O_2 uptake inhibition, per cent	
		Proline	Glutamate
$NaAsO_2$	50	73	97
	5	22	67
	1	9	0
$FeCl_3$	10	71	100
	5	5	48
	1	0	5
$ZnSO_4$	1	100	100
	0.5	44	69
	0.1	0	35
$NiSO_4$	10	86	93
	1	—	0
$CdSO_4$	10	90	100
	1	—	9
$CoSO_4$	10	3	51
$CuSO_4$	10	87	—
$BaCl_2$	10	—	14
PCMB	5	94	—

Accumulation of Glutamic Acid—When L-proline was oxidized by the cell suspension in the presence of various inhibitors, the accumulation of L-glutamate was demonstrated as shown in Table III. In the presence of 10^{-3} *M* per litre of $NaAsO_2$, the oxidation of proline resulted in the accumulation of glutamate and bisulfite binding compounds and the disappearance of proline and oxygen as shown in Table IV. As shown in the last column of the table the oxygen absorption was also calculated on the assumption that the main part of the bisulfite binding compounds (B) was the primary oxidation product of proline and the main part of the oxidation products present in the system, except for glutamate and B, was the last oxidation products (E), which were obtained from proline by the maximum absorption of 5.6 atoms of oxygen in the other experiment.

Glutamic γ-Semialdehyde as the Possible Direct Oxidation Product of Proline—In the presence of DL-glutamic γ-semialdehyde as substrate the cell suspension did not absorbe oxygen undr the condition where the oxygen absorption by proline and glutamate could be seen (Table V). However, the disappearance of the γ-semialdehyde without concomitant evolution of CO_2 were shown in this case. On the other hand, glutamic γ-semialdehyde was not detected paperchromatographically from the reaction mixtures of the proline oxidation (Table VI). Semicarbazide, hydroxylamine, phenylhydrazine, and *o*-aminobenzaldehyde were not effective as the possible trapping agents. In this paper chromatography the strongly ninhydrin positive spot (R_f value, 0.57 and 0.70–0.73, for the solvent A and B, respectively) except for glutamate was obtained and was largely different from glutamic γ-semialdehyde in the R_f values. It was confirmed from the isotopic experiment that glutamic γ-semialdehyde was

TABLE III

Accumulation of Glutamic Acid during Proline Oxidation

	Inhibitor		L-Glutamate found
Exp. I	$FeCl_3$	5×10^{-4} *M/litre*	0.02 μM/*ml.*
	$ZnSO_4$	5×10^{-5}	0.44
	$NaAsO_2$	5×10^{-3}	1.77
	$NaAsO_2$	1×10^{-3}	2.83
Exp. II	$FeCl_3$	8×10^{-4}	0.35
	$CoSO_4$	5×10^{-4}	1.05
	$ZnSO_4$	6×10^{-5}	0.61
	$NaAsO_2$	1×10^{-3}	1.07

Reaction mixture contained 30 μM (Exp. I) or 10 μM (Exp. II) of L-proline, 1 mM of NaCl, inhibitor as given in the table and cell suspension in a final volume of 1 ml. of 0.03 *M* veronal buffer pH 7.0. After shaking at 30° for 3 hours, L-glutamic acid was determined.

not the direct oxidation product of proline. Thus the uniformly C^{14}-labeled proline was oxidized by the cells in the presence of a large quantity of glutamic γ-semialdehyde and glutamate as trapping agents. If these were the oxidation products, these would be labeled after the oxidation reactions. As shown in the autoradiograms of Fig. 4, glutamic γ-semialdehyde was not labeled and glutamic acid was labeled even in the presence of glutamic γ-semialdehyde.

TABLE IV

Proline Oxidation in the Presence of 10^{-3} *M litre of* $NaAsO_2$

Reaction time (*hrs.*)	O_2 uptake (observed) (μ-atom/*ml.*)	Proline found decreased (μM/*ml.*)		Glutamate found (μM/*ml.*)	B-bompd.* found (μM/*ml.*)	O_2 uptake (calc.) (μ-atom/*ml.*)
0	0	9.7_0	—	—	—	0
2	8.2_3	6.9_4	2.7_6	1.3	0.46	8.6_6
4	16.2	5.6_1	4.0_9	2.2	1.16	15.2
4 (no arsenite)	36.7	1.8_7	7.8_3	0.7_6	0.82	$37._3$

* B-compd. : Bisulfite binding compounds.

Other composition of reaction mixture was the same as given in Table III.

TABLE V

The Oxidizing Activities of Cell Suspension for Various Substances

Substrate	O_2 uptake (*μl./3 hrs.*)
L-Proline	937
DL-Glutamic γ-semialdehyde	—5
L-Glutamic acid	622

DISCUSSION

That proline is metabolized *via* glutamic acid *in vivo* was shown by the finding of stably bound deuterium as well as N^{15} in glutamic acid isolated from the proteins of rat which has been fed doubly labelled L-proline (*12*). Initial dehydrogenation of proline on either side of the ring nitrogen atom, followed by hydrolytic opening of the ring, would result in formation of either α-keto-δ-aminovaleric acid or glutamic γ-semialdehyde. The alternative possibilities are indicated in Diagram

1. α-Keto-δ-aminovaleric acid was isolated after the oxidation of L-proline by a kidney or liver L-amino acid oxidase preparation of Green, which was completely free of D-amino acid oxidase activity (*13*). The resulting α-keto-δ-aminovaleric acid might undergo oxidative deamination to the semialdehyde of α-ketoglutaric acid, then further oxidation to α-keto-glutaric acid, which secondarily, through transamination, would yield glutamic acid. Taggart and Krakaur found, on the other hand, that a preparation of washed insoluble particles from homogenized rabbit kidney was capable of rapid and complete oxidation of proline under conditions which resulted in no disappearance of α-keto-δ-aminovaleric acid, and under incomplete conditions accumulated a carbonyl compound which was presumably the semialdehyde of glutamic acid (*14*). In *E. coli*, *N. crassa* and *T. utilis* the major paths of proline synthesis have all been shown by the mutant studies and isotopic competition experiments to involve the same sequence ; glutamate, glutamic γ-semialdehyde, Δ′-pyrroline-5-carboxylate, proline. The semialdehyde was found to be in equilibrium with Δ′-pyrroline-5-carboxylate (*15–18*).

TABLE VI

The Ninhydrin Positive Spots on the Paperchromatogram of the Reaction Mixture for the Proline Oxidation

Sample	R_f value of spots	
	n-Butanol-acetic acid-H_2O (A)	Phenol-H_2O (B)
I.	0.20, 0.41,* 0.57	0.28, 0.70
II.	0.11, (Y) 0.20, 0.41,* 0.57	0.28, 0.69, (Y) 0.73
Glutamic acid	0.20	0.28
Proline	0.32 (Y)	0.88 (Y)
Glutamic γ-semialdehyde	0.43	0.81
Semicarbazide	0.11 (Y)	0.69 (Y)

* These spots disappeared after 1 day.

Sample I : After the oxidation of 10 μM/ml. proline in 0.02 *M* phosphate buffer pH 7.0 and 1 *M* NaCl for 4 hours at 30°, the reaction mixtures were centrifuged.

Sample II : Reaction mixture the same as I., except for the presence of 0.01 *M* semicarbazide.

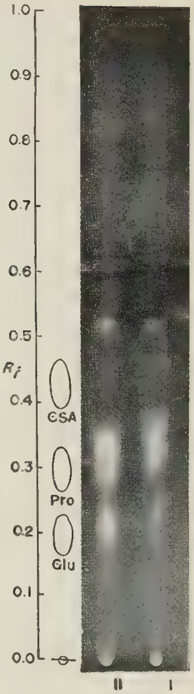
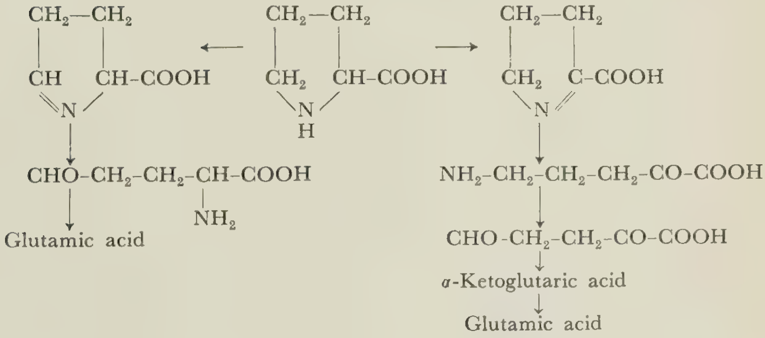


FIG. 4. Autoradiogram of Oxidation Products of Proline.

Reaction mixture I and II, contained 4× 10^4 c.p.m. of radioactive L-proline, 0.1 μM of L-proline, 1 μM of L-glutamate, 1 μM of DL-glutamic γ-semialdehyde (only in II) and cell suspension in a final volume of 1 ml. of 0.02 *M* phosphate buffer pH 7.0 and 1 *M* NaCl. After the reaction time of 1 hour at 30°, the reaction mixtures were centrifuged. The developing solvent in paperchromatography was *n*-butanol-acetic acid-water (4 : 1 : 1).

DIAGRAM 1

Alternative Possibilities in Oxidation of Proline



In the present study, halophilic bacterium No. 101 was capable of oxidizing L-proline and L-glutamic acid rapidly and accumulated L-glutamate in the former case, and not capable of oxidizing DL-glutamic γ-semialdehyde under the same conditions. This latter result cannot be ascribed to the permeability of this semialdehyde in the cell membrane, because of the finding of the considerable disappearance of this semialdehyde in the same time. D-Form of this semialdehyde might inhibit the enzymic oxidation of L-form. From the isotopic and paper-chromatographic experiments it is fairly clear that glutamic γ-semialdehyde is not produced as the direct intermediate when L-proline is oxidized by the cell suspension of this bacterium under the above described conditions.

In this bacterium proline would be oxidized *via* α-keto-δ-aminovaleric acid, if proline were oxidized only through one of the two known different pathways (Diagram 1).

Regarding the proline biosynthesis, only one path has been known: glutamate, glutamic γ-semialdehyde, proline. If so, L-proline would be required for the growth of this bacterium.

These suggestions would be confirmed by the further studies.

SUMMARY

1. Some properties of the L-proline oxidizing enzyme system of the cell suspension of a halophilic *Pseudomonas* strain No. 101 were studied.

2. Accumulation of L-glutamic acid was shown during the oxidation of L-proline.

3. Isotopic experiments and the oxidation test for glutamic γ-semialdehyde showed that this aldehyde might not be the direct intermediate in the oxidation of L-proline by this bacterium.

The author is indebted to Prof. S. Akabori for the helpful criticism and encouragement during the course of this work, to Prof. F. Egami, Nagoya University, for the gift of the bacterial strain, to Miss T. Wada for the gift of uniformly C^{14}-labeled L-proline, and to Mr. T. Okuda of the Laboratory for Protein Research of Osaka University for the gift of DL-glutamic γ-semialdehyde dimethylacetal.

REFERENCES

(*1*) Egami, F., *Bull. soc. chim. biol.*, **37**, 207 (1955)
(*2*) Flannery, W. L., *Bacteriol. Rev.*, **20**, 49 (1956)
(*3*) Shiio, I., Maruo, B., and Akabori, S., *J. Biochem.*, **43**, 779 (1956)
(*4*) Shiio, I., *J. Biochem.* **44**, 175 (1957)

(*5*) Chinard, F. P., *J. Biol. Chem.*, **199**, 91 (1952)
(*6*) *Organic Synthesis*, **28**, 11 (1948)
(*7*) Clift, F. P. and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932)
(*8*) Wada, T., Nomura, M., Mitsui, H., Maruo, B., and Akabori, S., *Radio-isotope*, **3**, 19 (1954)
(*9*) Wada, T., Nomura, M., Mitsui, H., Maruo, B., Tamiya, H., and Akabori, S., *J. Gen. Appl. Microbiol.*, **1**, 142 (1955)
(*10*) Olson, J. A. and Anfinsen, C. B., *J. Biol. Chem.*, **202**, 841 (1953)
(*11*) Land, K. and Schmid, G., *Biochem. Z.*, **322**, 1 (1951)
(*12*) Stetten, M. R., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 113 (1944)
(*13*) Blanchard, M., Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **155**, 421 (1944)
(*14*) Taggart, J. V., and Krakaur, R. B., *J. Biol. Chem.*, **177**, 641 (1949)
(*15*) Vogel, H. J., and Davis, B. D., *J. Am. Chem. Soc.*, **74**, 109 (1952)
(*16*) Abelson, P. H., *J. Biol. Chem.*, **206**, 335 (1954)
(*17*) Vogel, H. J., and Bonner, D. M., *Proc. Natl. Acad, Sci. U. S.*, **40**, 688 (1954)
(*18*) Vogel, H. J., Abelson, P. H., and Bolton, E. T., *Biochim. et Biophys. Act.*, **11**, 584 (1953)

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METABOLISM OF D-GLUCOSAMINE

II. THE FORMATION OF GLUCOSAMINIC ACID*

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(Received for publication, June 13, 1957)

The study on anaerobic metabolism of glucosamine (GA) has achieved a remarkable development in these several years and the conversion of GA to fructose-6-phosphate (F-6-P) *via* the preceding phosphorylation was verified (*1–5*). The present author considered a mechanism of the conversion as a sort of Lobry de Bruyn isomerization theory (6) and assumed the presence of a new enzyme " phosphoglucosamine isomerase."

In the early period of the study of GA metabolism, Lutwak-Mann (*7*) reported that aminosugars are first converted by oxidation into an unknown product (not glucosaminic acid (GAA)) which then undergoes deamination in mammal-bodies and bacteria. With regard to the oxidative metabolic pathway, there have been some other reports (*8–9*), but it is still obscure.

In this paper, the author describes the aerobic formation by *Pseudomonas fluorescence* (*Ps. fluor.*) of GAA from GA.

It has been reported by Imaizumi (*10*) that GAA is decomposed by mammals and bacteria.

The results presented in this paper show the presence of the aerobic degradation pathway of GA and the formation of GAA in normal metabolic patterns.

EXPERIMENTAL

All the materials and techniques except those described below were the same to those in the preceding paper (*3*).

MATERIALS

Standard GAA—Prepared from GA by oxidation with HgO (*11*).

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* Imanaga, Y., *J. Biochem.*, **44**, 397 (1957) Letters to the Editors ; Imanaga, Y., Sekiya, Y., Ogura, S., Mikami, M., and Matsushima, Y., reported at the 10th Annual Meeting of the Japanese Chemical Society (Tokyo University) in April (1957).

Ps. fluor.—Kindly supplied from Prof. Dr. M. Suda, Research Institute for Microbial Diseases, Osaka University, cultivated for two days at 25° and washed twice with distilled water. Cell-free extract was obtained by disintegration with silica sand.

Analytical Methods

Measurements of Gases Formed or Consumed—Warburg's manometric techniques were used. In anaerobic studies, air was replaced with nitrogen.

H_2O_2—To a test solution were added 5 ml. of 2 N H_2SO_4, 5 ml. of 1.5 M KI and 1 drop of 1 per cent ammonium molybdate and after 3 minutes I_2 was titrated with 0.01 N thiosulfate.

RESULTS AND DISCUSSION

(*1*) *Comparative Studies of the Degradation of Sugars by Various Species of Bacteria*—As shown in Table I, the amounts of O_2-uptakes by three species of bacteria were similar except for GA. But, *Ps. fluor.* consumed only one gram atom of oxygen per mole of GA and could not decompose it anaerobically, whereas the other two decomposed it similarly as common sugars both aerobically and anaerobically. These results show that there is a presence in *Ps. fluor.* of a different oxidative metabolic pathway of GA from the others (anaerobic degradation).

(*2*) *Reaction Products*—As shown in Fig. 1, the amounts of O_2-uptakes at pH 7,0 and 8,3 for a small amount of GA were equivalent to one gram atom and added GA disappeared almost completely. But, for a large amount of substrate, the amount of O_2-uptake did not reach one gram atom and a considerable amount of GA remained. The pH dropped to about 5,2 indicating the formation of some acidic substance and on readjustment of pH to 7,0 the O_2-uptake again started. *Ps. fluor.* could not decompose GA at pH 5,2.

From Fig. 2, the formation of GAA was clear, though, in (A), spots were not clear similarly as those of the standard run.

(*3*) *Isolation of GAA*—Scheme 1 shows the isolation procedure.

The crystals thus obtained showed the same chromatographic behavior with the authentic specimen (R_f=0,35 in (B)) and the good coincidence in nitrogen analysis (Found: 7,12 per cent, Theor.: 7,18 per cent, $C_6H_{13}O_6N$).

(*4*) *Stoichiometry of the Reaction*—After one gram atom of oxygen per mole of GA was absorbed, the reaction mixture contained scarcely any GA, but there remained strong reducing power against Somogyi's reagent. It was an unexpected fact that GAA reduced Somogyi's reagent quantitatively (Fig. 3).

By combining this reducing reaction with Elson-Morgan's color reaction and manometric measurements of O_2-uptakes, the stoichiometry of the reaction was studied both in intact cell and in cell-free extract (Table II). The results may be expressed in equation (1).

TABLE I

The Degradation of Sugars by Three Species of Bacteria

Substrate \ Species	Soil bacteria* at 30°, pH 6.0	*A. cloacea* at 37°, pH 7.0	*Ps. flucr.* at 30°, pH 7.0
Aerobic degradation			
	Gram atoms of O_2-uptake per mole of substrate		
Glucose	*ca.* 6 (3)**	*ca.* 7–8	*ca.* 7
Fructose	6	8	7–8
Gluconic acid	5	5	6
GA	5–6 (3)**	7–8 (3)**	1 (0)**
Anaerobic degradation			
Glucose	(1,2)** H_2‡; 1,2 mole		
GH	(1,5)** H_2‡; 1,2 mole	H_2‡; 0,7 mole	Not degraded

4/3 μM or 8/3 μM of substrate and phosphate buffer of about *M*/2 concentration were used.

* Isolated by the author and cultivated at 25°. This produced 2,3-butyleneglycol similarly as *A. cloacea* (*6*) from GA.

** ( ) indicates moles of CO_2 formed per mole of substrate.

‡ Probably hydrogen (formed per mole of substrate), being not absorbed by KOH.

$$GA + 1/2\ O_2 + H_2O \longrightarrow GAA + H_2O \qquad (1)$$

Because of the co-existence of powerful catalase (Fig. 4), the O_2-uptake of one gram atom per mole of GA in this reaction seems to be resulted from the secondary action of catalase according to equation (2) and (3).

$$GA + O_2 + H_2O \xrightarrow{\text{Enzyme}} GAA + H_2O_2 \qquad (2)$$

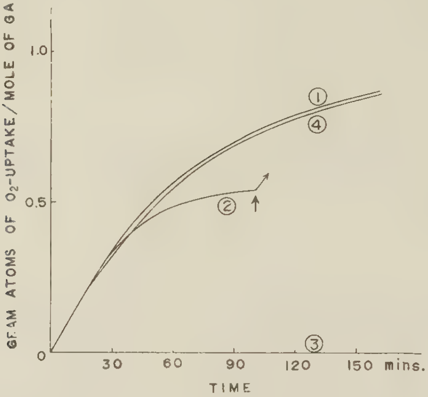

FIG. 1. Effect of pH on the degradation of GA by *Ps. fluor.*
Conditions : all was carried in phosphate buffer.

① : 8/3 μM of GA and pH 7.0 (concentration of buffer, 0.042 *M*), ② : 1 mM of GA and pH 7.0 (concentration of buffer, 0.029 *M*). At the point (↑), pH was re-adjusted to 7.0, ③ : 8/3 μM of GA and pH 5.2 (concentration of buffer, 0.025 *M*), ④ : 8/3 μM of GA and pH 8.3 (concentration of buffer, 0.042 *M*).

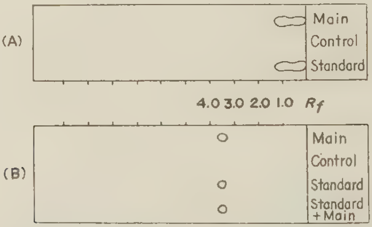

FIG. 2. Paperchromatograms of the reaction product.

1 mM of GA (2.0 ml., adjusted to pH 7.0) was shaken with 37 ml. of washed cell suspension of *Ps. fluor.* in phosphate buffer (pH 7.0, final concentration: 0.145 *M*) using a large Warburg vessel for about 3 hours at 30°. The amount of O_2-uptake was one gram atom permole of GA and GA was almost completely consumed. After centrifuging cells, the supernatants were concentrated *in vacuo*. Ninhydrin was used as a developer. Moving phase system ; (A): Phenol-water (4 : 1), (B): *t*-Butanol-formic acid-water (3 : 1 : 1). The standard run is authentic GAA dissolved in phosphate buffer (pH 7.0).

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + 1/2\ O_2 \qquad (3)$$

$$GA+1/2O_2+H_2O \longrightarrow GAA+H_2O \qquad (1)$$

Scheme 1

Isolation Procedure of GAA

Doubly combined supernatant of main experiment in Fig. 2.
(Degradation of GA ,99%)
| added with $Ba(OH)_2$ solution, until no precipitate was formed and centrifuged. Precipitate was washed with water and combined to the supernatant.
↓ Barium phosphate
↓ Supernatant
| added with H-type of Amberite IRC-50, until pH of the solution dropped to 6,7, centrifuged, the resin was washed with water and combined to the supernatant.
↓
Supernatant
↓ evaporated to dryness *in vacuo.*
Residue
| recrystallized twice from water with charcoal
↓ decolorization.
White crystals (GAA)
150 mg.

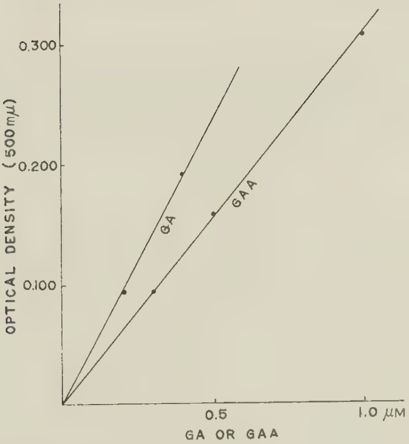

Fig. 3. Somogyi's reaction* of GA and GAA
* Carried out at 100° for 25 minutes.

TABLE II

Stoichiometry of the Reaction

	GA added (μM)	O_2-uptake (μM)	GA disappeared (μM)	GAA formed (μM)
Cell-free extract	8,0	3,6	7,3	7,2*
Intact cell	8,0	4,0	7,8	8,2

8 μM (0.4 ml.) of GA was incubated at 30° with 3.4 ml. of intact cell suspension or cell-free extract (dialysed for about 15 hours) in phosphate buffer (pH 7.0, final concentration: 0.025 *M*) in Warburg vessel with 0.2 ml. of 10 per cent KOH in a center well. After the O_2-uptake ceased, cells or proteins precipitated with 0.5 ml. of 10 per cent metaphosphoric acid were centrifuged. The supernatants thus obtained were assayed as usual.

* Identified chromatographically, also.

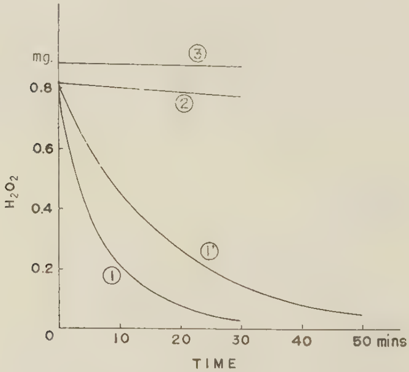

FIG. 4. Catalase activities of intact cell suspension and cell-free extract.

Conditions : all the procedure was carried at 0°.

① : 4 ml. of about 0.22 per cent H_2O_2 was mixed with 46 ml. of *M*/15 phosphate buffer (pH 7.0), then 0.1 ml. of crude extract was added. ② and ③ : 8.9 ml. of phosphate buffer, 0.1 ml. of crude extract and 1.0 ml. of *M*/20 inhibitor (② : Na_2S, ③ : NaN_3) were mixed. After a while, 36 ml. of phosphate buffer and 4.0 ml. of H_2O_2 were added. (Final concentration of inhibitor: 10^{-3} *M*). ①′ : In ①, 1 ml. of cell suspension (2.66 mg. dry weight) was used in place of extract. Each 5 ml. of above mixtures was assayed at time intervals.

If truly so, when the reaction (3) is neglected by some treatment, the O_2-uptake will rise to one mole per mole of substrate and the reaction will become analogous to the conversion of glucose to gluconic acid by Notatin (*12*). Further studies are on progress.

(*5*) *Effect of Addition of Several Chemicals to the Dialysed Extract*—The effect of several additions to dialysed or acid treated (precipitation at pH 5.0) and dialysed extract were studied. FAD*, TPN*$+Mg^{++}+$MB*, Mg^{++}, Fe^{+++}, EDTA*, PCMB*, NaF, DNP*, $I.CH_2{\cdot}COOH$, AsO_4^{---} and AsO_3^{---} were all without effect. But, CN^- and NH_2OH fairly suppressed and Cu^{++} inhibited almost completely the O_2-uptake at the concentration of 10^{-3} *M*.

The effect of CN^- and NH_2OH may be due to their reaction with the aldehydic group of GA and that of Cu^{++} due to the reaction with the enzyme.

SUMMARY

1. Intact cell of *Ps. fluor.* and cell-free extract of it were capable of converting GA to GAA according to the equation,

$$GA + 1/2\ O_2 + H_2O \longrightarrow GAA + H_2O$$

2. From the reaction mixture by intact cell, GAA was isolated.

3. CN^- and NH_2OH fairly and Cu^{++} almost completely inhibited the O_2-uptake.

The author takes liberty of expressing his thanks to Prof. Dr. S. Akabori of Osaka Univ. and Prof. Dr. Y. Matsushima for their kind guidance, and also to misses Y. Sekiya, S. Ogura and M. Mikami for their assistance.

REFERENCES

(*1*) Leloir, L. F., and Cardini, C. E., *Biochim. Biophys. Acta*, **20**, 33 (1956)

(*2*) Imanaga, Y., *J. Biochem.*, **44**, 69 (1957) ; Imanaga, Y., Kusanagi, Y., Yama-

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* FAD (Flavinadeninedinucleotide) and
TPN (Triphosphopyridinenucleotide) ; kindly supplied by Prof. Dr. K. Okunuki, Osaka Univ.
MB (Methyleneblue) ;
EDTA (Ethylenediamine tetraacetate) ;
PCMB (*p*-Chloromercuribenzoate) ;
DNP (2,4-Dinitrophenol).

da, Y., Maeda, K., Abu, M., and Matsushima, Y., Reported at the Symposium on Enzyme Chemistry (Tokyo Univ.), July (1956)

(*3*) Faulkner, P., and Quastel, J. H., *Nature*, **177**, 1216 (1956)

(*4*) Comb, D. C., and Roseman, S., *Biochim. Biophys. Acta*, **21**, 193 (1956)

(*5*) Yamaha, T., and Asahina, M., *J. Jap. Biochem. Soc.*, **28**, 486 (1956)

(*6*) Matsushima, Y., *Sci. Pap. Osaka Univ.*, No. 34 (1951) ; *Chemisches Zentralblatt*, **124**, 1489 (1953)

(*7*) Lutwak-Mann, C., *Biochem. J.*, **35**, 610 (1941)

(*8*) Tadokoro, T., and Saito, T., *J. Agr. Chem. Soc. Japan*, **18**, 394, 1159 (1942)

(*9*) Rosenberg, A. J., *Compt. rend.*, **226**, 1751 (1948)

(*10*) Imaizumi, M., *J. Biochem.*, **26**, 197 (1937)

(*11*) Pringsheim, H., and Ruschmann, G., *Ber.*, **48**, 681 (1915)

(*12*) Coulthard, C. E., Michaelis, R., Short, W. F., Slykes, G., Skirmshire, G. E. H., Standfast, A. F. B., Birkinshow, J. H., and Raistrick, H., *Nature*, **150**, 634 (1942) ; *Biochem. J.*, **38**, 24 (1945)

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QUANTITATIVE DETERMINATION OF THIAMINE PYROPHOSPHATE USING APOCARBOXYLASE AND ALCOHOL DEHYDROGENASE*

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(Received for publication, July 2, 1957)

The enzymic methods for the quantitative determination of TDP**, *i.e.*, cocarboxylase, are based on its action as a cofactor for the enzyme carboxylase. The most commonly used procedure is to measure manometrically the carbon dioxide liberation from pyruvate after recombination of the apoenzyme with TDP. Under appropriate conditions, this quantity of carbon dioxide evolved may be used as the quantitative measure of the amount of TDP (*1–5*), but the difficulty associated with this procedure is due to the fact that free thiamine has sometimes a marked stimulatory effect on the action of TDP (*2*). Moreover, other substances, which might cause side reactions resulting in a non-specific evolution of carbon dioxide, might interfere in the determination.

Holzer (*6*) described in 1953 a new assay method for carboxylase, in which the enzyme activity was estimated by determining acetaldehyde instead of carbon dioxide formed in the reaction (a).

In the presence of ADH, as is shown in equation (b), this acetaldehyde was reduced to alcohol by DPNH, the disappearance of which was followed spectrophotometrically at 340 mμ.

$$CH_3COCOOH \xrightarrow{\text{carboxylase}} CH_3CHO + CO_2 \qquad (a)$$

$$CH_3CHO + DPNH + H^+ \xrightarrow{\text{ADH}} CH_3CH_2OH + DPN^+ \qquad (b)$$

The author tried to apply this assay method to the determination

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[*] This paper was presented at the 9th General Meeting of Japan Vitamin Society held in May 1957, at Osaka.

[**] The following abbreviations are used : TDP for thiamine pyrophosphate, ADH for alcohol dehydrogenase, DPN or DPN$^+$ for diphosphopyridine nucleotide, DPNH for reduced DPN, KG for α-ketoglutarate, EDTA for ethylenediaminetetraacetic acid, and R-5-P for ribose-5-phosphate.

of TDP, so as to avoid the foregoing difficulties in the manometric method. To the apocarboxylase prepared as described below, Mg ions, sodium pyruvate, DPNH, yeast ADH, and varying amounts of TDP were added, so that the above two reactions took place, in which the reaction (a) was always rate limiting, and the determination was made by estimating the rate of decrease in DPNH absorption at 340 mμ.

By this procedure, it has become possible to determine far minute quantities of TDP, and to eliminate other side reactions. Besides, the technique is very simple and the results are highly reproducible.

METHOD

Materials—Pyruvic acid was chemically synthesized from tartaric acid (*7*), and neutralized with NaOH by the method of Robertson (*8*). Standard TDP solution was freshly prepared by dissolving TDP, which was supplied from the Takeda Pharmaceutical Industries, Ltd., Japan (Purity ; 91.2 per cent ionophoretically, 91 per cent enzymically). DPN, approximately 75 per cent pure, was obtained from the Sigma Chemical Company and was reduced chemically with dithionite (*9*).

Enzymes—(*A*) *Apocarboxylase solution:*

1) *Purification of Carboxylase*—Carboxylase was prepared by the method of Green *et al.* (*10*), and Uehara *et al.* (*11*), modified as follows : 100 g. of dried baker's yeast* was autolysed and extracted with 300 ml. of 0.1 *M* disodium phosphate at 37° for about 150 minutes with occasional stirring. After centrifugation the residue was washed with 300 ml. of 0.1 *M* disodium phosphate, the washings were combined with the original extract, and the total solution was filtered with suction through a large Buchner's funnel employing 100 g. of Hyflo Super-Cel. The almost clear filtrate was obtained. The subsequent steps were carried out at 0°. To this filtrate 80 ml. of 0.5 *M* phosphate buffer (pH 7.2) and 40 ml. of 1 *M* calcium acetate were added with mechanical stirring, and the resulting precipitate was removed by centrifugation. For every 100 ml. of the supernatant 35 g. of solid ammonium sulfate was added and this suspension was left overnight in a refrigerator at 0°. After centrifugation the precipitate was washed once with 200 ml. of ammonium sulfate solution (0.55 saturation, pH 6.0), and then suspended in 200 ml. of dilute ammonium sulfate solution (0.38 saturation, pH 6.0). The protein precipitating at this ammonium sulfate saturation was discarded after centrifugation. The proteins in the supernatant were precipitated by the further addition of 24 g. of solid ammonium sulfate with stirring, and this mixture was left for 30 minutes at 0° before centrifugation. The precipitated protein was then dissolved in 50 ml. of 0.5 *M* citrate buffer (pH 6.0) and used as the carboxylase holoenzyme solution. Stored at −4°, this preparation retained its activity for several weeks.

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* The fresh baker's yeast was obtained from the Oriental Yeast Company (Japan) and dried in the usual manner.

2) *Spectrophotometric Assay of Enzyme Activity*—During the purification, the enzyme activity was checked spectrophotometrically by the method of Holzer (*6*), modified as follows : the assay system contained in 3.5 ml. quartz cell ; 0.6 ml. of *M*/6 citrate buffer pH 6.0, 0.1 ml. of 0.5 per cent DPNH solution, 0.1 ml. of 1.5 *M* sodium pyruvate, 0.1 ml. of yeast ADH (0.2 mg), and 2.0 ml. of distilled water. The reaction was started by the addition of 0.1 ml. of the carboxylase preparation and the decrease in optical density at 340 mμ was measured spectrophotometrically. The amount of enzyme added to the assay system was chosen so that the rate of decrease in optical density might be 0.020 to 0.100 per minute. Protein determinations were carried out according to Warburg and Christian (*12*)

3) *Preparation of Resolved Enzyme* (*10*)—To the above carboxylase solution were added two volumes of chilled water and 4 volumes of ammoniacal ammonium sulfate solution (9 parts of saturated ammmonium sulfate to one part of conc. ammonia), pH was adjusted to 9.0 with conc. ammonia, and the solution was left overnight at 0°. The precipitate was collected by centrifugation at 3,000 r.p.m. for 30 minutes, and washed once with ammoniacal ammonium sulfate solution (0.52 saturation, pH 9.0), and finally dissolved in 50 ml. of 0.5 *M* citrate buffer, pH 6.0. The solution was centrifuged and adjusted to pH 6.0 with dilute H_2SO_4. This solution was almost colorless, and the apoenzyme was completely freed of the coenzyme. As described below, it was sufficiently pure for the purpose of TDP determination. The apoenzyme, frozen and stored at −4°, retained its activity for several months.

(*B*) *Alcohol Dehydrogenase :*

ADH was isolated and purified from baker's yeast by the method of Racker (*13*). A small residual carboxylase activity in the ADH preparation might be due to contamination with carboxylase and was eliminated simply by Norit treatment, adsorbing the TDP from alkaline solution of the enzyme preparation in the following manner : an ADH solution containing about 100 mg. of protein, was diluted to 20 ml. with distilled water, and was adjusted to pH 9.0 with conc. ammonia. About 100 mg. of Norit was added and the mixture was stirred for 10 minutes. The Norit was centrifuged off, and 36 g. of solid ammonium sulfate was added for every 100 ml. of the supernatant solution. After standing at 0° for 30 minutes, the mixture was centrifuged at 15,000 r.p.m. for 20 minutes at 0°. The precipitate was dissolved in 8 ml. of distilled water, and after centrifugation at 15,000 r.p.m. for 10 minutes in a refrigerated centrifuge, the supernatant was freed of carboxylase activity. Any floating material was removed by filtration and 2 ml. of saturated ammonium sulfate solution was added to the resulting clear solution. This suspension keeps for about 4 weeks at −4°.

For the carboxylase assay system, this suspension was diluted five times so as to contain 0.2 mg. of protein in 0.1 ml.

Procedure—The composition of the standard reaction mixture for quantitative determination of TDP was as follows : citrate buffer 100 μM, pH 6.0 ; DPNH, 0.5 mg. ; sodium pyruvate, 150 μM ; carboxylase-free ADH, 0.2 mg. ; Mg ions, 120 μg. ; varying amounts of TDP ; apocarboxylase, 0.42 mg. ; and distilled water to final volume of 3.0 ml. Apoenzyme, Mg ions and TDP were added in the order named and the reaction mixture, except pyruvate, allowed to stand for 10 minutes at room temperature in order to recombine TDP with apoenzyme and Mg ions, then the reaction was started by the addition of pyruvate. When larger amounts of TDP (more than 60 mμg) were to be estimated, it could be proceeded alternatively in the following way ; apoenzyme, Mg ions and TDP were incubated in a test tube, and after the resynthesis of holocarboxylase had been completed, a suitable aliquot was transfered to the cell in which all the other components had been already placed. Decreaese in optical density at 340 mμ was followed spectrophotometrically using a quartz cell with 1.0 cm. optical path, at 17°. The readings were made against a blank which contained all the components except DPNH, and a control sample contained all the components except the substrate. The readings obtained with the complete system were always corrected by subtracting the readings in the control, but usually, when either pyruvate or TDP was omitted from this system, the reaction did not take place at all.

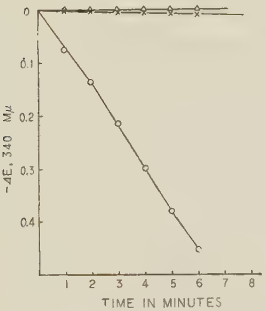

FIG. 1. Assay of carboxylase. The complete system contained : citrate buffer, 100 μM, pH 6.0 ; sodium pyruvate, 150 μM ; DPNH, 0.5 mg. ; carboxylase-free ADH, 0.2 mg.; carboxylase preparation, 78 μg.; and redistilled water to final volume of 3.0 ml. Reaction was started by the addition of carboxylase. —○— Complete system ; —△— without pyruvate ; —×— without carboxylate.

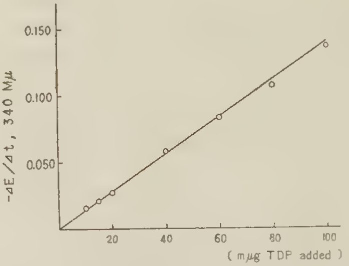

FIG. 2. Standard curve for TDP determination. The composition of the reaction mixture was as follows : citrate buffer, 100 μM, pH 6.0 ; sodium pyruvate, 150 μM ; DPNH, 0.5 mg. ; carboxylase-free ADH, 0.2 mg.; Mg ions, 120 μg. ; apocarboxylase 0.42 mg.; varying amounts of TDP, final volume 3.0 ml.

RESULTS AND DISCUSSION

Assay of Carboxylase Holoenzyme Preparation—The activity of 0.078 mg.

of holocarboxylase preparation, under the conditions described in "Method", is shown in Fig. 1. The decrease in optical density at 340 mμ proceeded at a constant rate after a short lag period. When either pyruvate or the enzyme was omitted from this system, the oxydation of DPNH was not observed.

Standard Curve—The standard curve is shown in Fig. 2. Under the standard conditions of the assay a linear proportionality was obtained between the rate of decrease in optical density at 340 mμ and the amount of TDP added in a range of 5 to 60 mμg. When TDP was omitted, the reaction did not occur.

Time and Temperature—The reaction proceeds linearly with time, but at the end of the reaction, the velocity decreases gradually. When the reaction velocity is very small, it proceeds at a constant rate for about 20 minutes or more. The determination was made at 17° only from practical reasons, and the amount of 5 mμg. of TDP could be estimated at this rather low temperature. As yeast carboxylase is reported to be three times more active at 37° (*15*), the sensitivity could be increased by the use of a higher temperature.

*Effect of Buffer**—According to Green *et al.* (*10*), carboxylase is most active in citrate buffer at pH 6.0. It was also found in the present spectrophotometric assay procedure that the reaction rate in the citrate buffer was almost twice as large as that in the phosphate buffer of the same pH (Fig. 3). Kay *et al.* (*5*) reported that their carboxylase preparation showed a very low activity in phosphate buffer and the addition of cyanide, thiamine, or citrate markedly stimulated the carbon dioxide evolution in their system. They attributed this phenomenon to the protection of SH groups of the enzyme protein against some interfering substances, probably a trace of metals. To examine this possibility 0.2 μM of EDTA was added to each of these two buffers. The reaction velocity was slightly increased but, the difference between these two buffers was still remarkable and therefore, the stimulatory effect of the citrate buffer seemed to be related to some anionic effect of citrate.

Effect of Pyruvate Concentration—It was reported that the enzyme was saturated at about $M/6$ concentration of pyruvate and half saturated at $3 \times 10^{-2}\ M$ (*10*). In the present spectrophotometric method, the effect of pyruvate concentration on the reaction velocity was examined and results similar to those of Green *et al.* (*10*) were obtained. Up to

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* In several of the following experiments, holocarboxylase preparation was used in place of apocarboxylase plus TDP and Mg ions.

0.05 M the rate of the reaction largely depended upon the pyruvate concentration, but, above this, there was no more marked difference (Fig. 4). Therefore, the determination was carried out at $M/20$ concentration of pyruvate in the present study.

Apocarboxylase—Most of the accepted methods employ alkali-washed yeast (etiozymase) (*1–4*), or somewhat purified apocarboxylase as the apoenzyme (*5*). However, these are not reliable sources of apoenzymes, since it is known these crude preparations are activated by the added thiamine (*2*). Besides, with some substances contained in tissue preparations, they have side reactions which could result in spurious evolution of carbon dioxide. Kay *et al.* (*5*), using a purified apocarboxylase prepared by their modification of the method of Green *et al.*, still found that cyanide, thiamine, and other substances markedly stimulated the carbon dioxide evolution in their system for TDP determination. It was therefore decided to prepare a more purified apoenzyme in the present study in order to produce a better assay system.

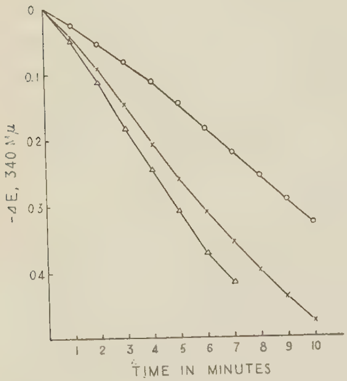

Fig. 3. Effect of buffer. —△— Citrate buffer $M/30$, pH 6.0 ; —×— citrate buffer $M/50$, pH 6.0 ; —○— phosphate buffer $M/50$, pH 6.0. Other conditions were the same as those in Fig. 1.

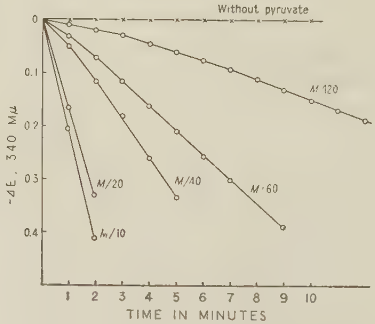

Fig. 4. Effect of pyruvate concentration. The conditions were as described in Fig. 1, except for pyruvate concentration as indicated.

Throughout the experiments 0.42 mg. of apocarboxylase, prepared by the purification procedure described under " Method ", was used. When this amount of enzyme was saturated with excess of TDP, it showed such a large activity that it was hardly measurable even after five fold dilution. When the amount of apoenzyme was increased to 0.61 mg., the reaction proceeded almost at the same rate, as in the case with 0.42 mg. of apoenzyme.

Mg Ions—120 μg. of Mg ions was used, this quantity being 220 times theoretical requirement for 0.42 mg. of apoenzyme.

Yeast Alcohol Dehydrogenase—ADH was prepared according to Racker (*13*). After the first ammonium sulfate fractionation, a small residual carboxylase activity was eliminated as described under " Method ".

The use of Norit for removing TDP by adsorption from alkaline solution was first introduced by Kay *et al.* (*5*) in their purification procedure of apocarboxylase. This technique was found to be very useful in the present experiments to eliminate this residual carboxylase activity in the ADH preparation. After this treatment the enzyme showed a negligible activity of carboxylase. This technique was also used to prepare the resolved carboxylase, but in this case it was found that the method described above seemed to be more preferable to that of Kay *et al.*, because of its better yield and its lesser extent of protein denaturation. In the case of ADH the enzyme protein might be inactivated when it was left overnight at pH 9.0 and the Norit treatment had the advantage of eliminating all the residual activity of carboxylase by only one treatment and of avoiding protein denaturation as far as possible.

In each experiment, 0.2 mg. of this carboxylase-free ADH was used, this amount being fairly large excess, which was capable of oxidizing all the DPNH added almost instantaneously in the presence of acetaldehyde.

Effect of Other Substances—Pyruvic carboxylase is known to act on a variety of organic acids containing a carbonyl group adjacent to a carboxyl group. Green *et al.* (*10*) and Kobayasi (*14*) made a comparative study on the rate of decarboxylation of various substrates by purified carboxylase and by crude yeast preparation, respectively.

The present assay method is not adequate to examine the specificity of the carboxylase preparation, as it is composed of two enzyme systems. For example, when one substrate fails to oxidize DPNH in the present system, it may be due to the fact that either this substrate is not decarboxylated by carboxylase, or the resulting aldehyde does not participate in the oxidation of DPNH catalyzed by the ADH system. However, as far as the determination of TDP is concerned, this method has an advantage because only those substances which could be decarboxylated by carboxylase and subsequently react with ADH system to oxidize

DPNH, could influence on the determination.

When alkali-washed yeast is used as the apoenzyme source for manometric determination of TDP, thiamine, cyanide, glucose, and other substrates on the glycolytic pathway might cause spurious evolution of carbon dioxide and thus make the accurate determination of TDP impossible. A number of procedures have been devised to avoid, inaccuracies associated with such difficulties. In the case of thiamine Westenbrink *et al.* (*16, 17*) have concluded that thiamine acts by inhibiting yeast phosphatase which dephosphorylates TDP, and Ochoa *et al.* (*2*) stated that it was therefore necessary to employ the apoenzyme activated by the excess of thiamine, in order to eliminate such stimulatory effect of thiamine. However, in these methods devised to determine the quantity of TDP in animal tissues which are known to contain ATP and other nucleoside polyphosphates in sufficient quantity to allow TDP synthesis from thiamine in the presence of thiaminokinase, it seems inappropriate to include excess of thiamine. The addition of monoiodoacetic acid was introduced by Blanchaer and Cohen (*18*), to prevent the pyrophosphorylation of thiamine, and some also used this acid to inhibit the carbon dioxide evolution from glycolytic substrates. But as there are evidences that carboxylase activity depends on intact SH groups, it might be inhibited itself by the addition of monoiodoacetic acid.

In the present study it was therefore decided to use a more purified apoenzyme preparation to avoid such side reactions. The results obtained with the apoenzyme purified by the author were as follows:

(1) Thiamine: In spectrophotometric assay system, the addition of 20 μg. of thiamine hydrochloride resulted in a slight inhibition. No stimulatory effect of thiamine was observed.

(2) Cyanide : As to the effect of cyanide, the present finding was entirely different from that reported by Kay *et al.* The oxidation of DPNH was completely inhibited in the presence of 3.3×10^{-3} M of cyanide, by which they found a marked stimulatory effect, and in a lower concentration (10^{-5} M), neither activation nor inhibition was observed. The inhibitory action of cyanide probably depends on its interaction with carbonyl group of pyruvate molecule or with aldehyde group of acetaldehyde formed, because, even when the reaction was inhibited by the presence of cyanide in a high concentration, further addition of acetaldehyde always caused the entire disappearance of the absorption of DPNH almost instantaneously.

(3) Glucose and lactate: The effect of glucose and lactate was

investigated as the substrates of glycolytic pathway. At first, 150 μM of glucose was added to the system in the absence of pyruvate, but the optical density at 340 mμ remained unchanged unless supplemented with pyruvate. When both glucose and pyruvate were added to the system from the beginning of the reaction, it proceeded practically at the same rate as in the control cell, to which glucose was not added (Fig. 5).

If carboxylase preparation was contaminated with lactic dehydrogenase system, it might oxidize lactate to pyruvate or reduce pyruvate to lactate, and thus might interfere in the determination of TDP. In order to test this possibility the effect of 150 μM of sodium lactate was also investigated. The results were almost the same as those with glucose (Fig. 6).

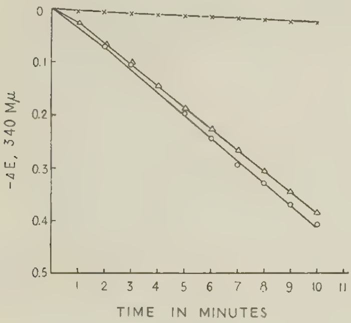

Fig. 5. Effect of glucose.
—○— control experiment; —△— control plus glucose, 150 μM; —×— pyruvate was omitted and 150 μM of glucose was added. Other conditions were the same as those in Fig. 2.

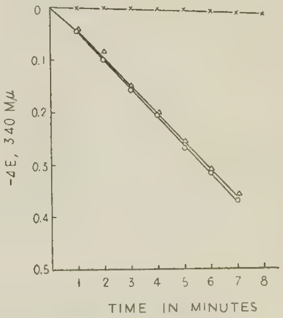

Fig. 6. Effect of lactate.
—○— control experiment; —△— control plus lactate, 150 μM; —×— pyruvate was omitted and 150 μM of lactate was added. Other conditions were the same as those in Fig. 2.

As for the lactic dehydrogenase system, yeast enzyme is reported to differ from muscle enzyme in being independent of pyridine nucleotide coenzyme. Therefore, there is no possibility that the determination might be interfered by coupled oxidation of DPNH with pyruvate, even when lactic dehydrogenase system was included in this enzyme preparation.

(4) Oxalacetate: This method is based on the measurement of DPNH oxidation by acetaldehyde in the presence of ADH. Consequently, there is a possibility of interferance by substrates of other dehydro-

genase systems which require DPN as a coenzyme. The effect of oxalacetate was investigated under such consideration and also for studying substrate specificity of this system for α-keto acid, but, as shown in Fig. 7, oxalacetate was reduced by the residual malic dehydrogenase activity in ADH preparation with the concomitant oxidation of DPNH, before the addition of carboxylase.

However, when TDP in animal tissues is determined, reaction mixture is preincubated for 10 minutes before the determination, in order to recombine the resolved enzyme with Mg ions and TDP. During this period, oxalacetate, which might be involved in tissue preparations, would be completely reduced by DPNH. Therefore, TDP can be estimated without any interference, when the reaction mixture is supplemented with proper amount of DPNH, previous to the addition of pyruvate to start the reaction.

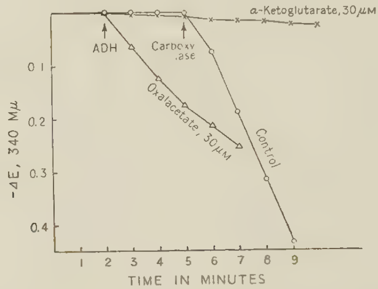

Fig. 7. Effect of α-ketoglutarate and oxalacetate. The conditions in control cell were the same as described in Fig. 1. —△— 30 μM of oxalacetate ; —×— 30 μM of α-ketoglutarate were added in place of pyruvate. In this experiment, ADH and carboxylase were added as indicated by the arrows.

(5) α-Ketoglutarate : 30 μM of KG was added in the presence or in the absence of pyruvate to see whether it might participate in the assay system. However, KG did not oxidize DPNH in this system and had no effect on the reaction when pyruvate was also present.

(6) R-5-P : The assay of enzyme transketolase which is recognized to require TDP for its activity, is also carried out spectrophotometrically. The principle of this method (*19*) is as follows : R-5-P added as substrate undergoes following five reaction sequences ((a) isomerization of R-5-P to ribulose-5-P, (b) epimerization to xylulose-5-P, (c)

transketolation between xylulose-5-P and R-5-P to form sedoheptulose-7-P and glyceraldehyde-3-P, (d) isomerization of glyceraldehyde-3-P to dihydroxyacetone phosphate, and (e) reduction to α-glycerophosphate), and in the last step catalyzed by α-glycerophosphate dehydrogenase, dihydroxyacetone phosphate is reduced to α-glycerophosphate by DPNH, the disappearance of which is measured spectrophotometrically at 340 mμ.

The reaction (c) is catalyzed by the enzyme transketolase. In case the apocarboxylase is contaminated with the above five enzymes and, furthermore, a considerable amount of R-5-P is involved in tissue extracts to be assayed for TDP, R-5-P might interfere with the apocarboxylase system against TDP determination. These seem to be highly improbable, yet, as this possibility could not be entirely excluded, the effect of the addition of R-5-P was investigated. The rate of the reaction was followed during a fairly long time, in the presence of 3.6 μM of R-5-P and it was proved to be inactive.

(7) Acetaldehyde: Acetaldehyde is known as an effective inhibitor of carboxylase (*1*, *10*, *20*). In fact, when manometric method was used, the rate of the enzyme reaction therefore decreased with time as the reaction product accumulated, but in the present system, it was immediately reduced to ethanol so that it could not exhibit any inhibitory action.

It may therefore be concluded that in the spectrophotometric method with the purified apoenzyme, the substances investigated above do not interfere in the determination of TDP, except oxalacetate which is reduced to malate in this system.

Note. Since this paper accepted for publication, the investigation of Holt *et al.* (*21*) has been reported in which the determination of TDP was also made spectrophotometrically using ADH.

SUMMARY

1. A new spectrophotometric method suitable for determination of TDP is presented.

2. The purification procedures of apocarboxylase and alcohol dehydrogenase from baker's yeast for this purpose are described.

3. This method enables determination of 5 to 60 mμg. of TDP at 17°, and it is expected to be more sensitive when carried out at a higher temperature.

4. The presence of glucose, lactate, R-5-P, and KG did not inter-

fere in the accurate determination of TDP by this method.

5. Thiamine did not exhibit any stimulatory effect on the reconstracted carboxylase preparation employed in this experiment.

The author wishes to express the heartful gratitude to Prof. N. Shimazono for his helpful advices and continuous encouragement.

REFERENCES

(*1*) Lohmann, K., und Schuster, P., *Biochem. Z.*, **294**, 188 (1937)

(*2*) Ochoa, S., and Peters, R. A., *Biochem. J.*, **32**, 1501 (1938)

(*3*) Westenbrink, H. G. K., and Steyne-Parvé, E. P., *Int. Z. Vitaminforsch.*, **21**, 461 (1949)

(*4*) Horié, S., *J. Vitaminology* (*Japan*), **3**, 1 (1957)

(*5*) Kay, W. W., and Murfitt, K. C., *Biochem. J.*, **62**, 601 (1956)

(*6*) Holzer, H., 4 Colloquium der Gesellschaft für physiol. Chemie, S. 89–114, Springer. (1953)

(*7*) Organic Synthesis, Coll. Vol. **1**, 475

(*8*) Robertson, W. B., *Science*, **96**, 93 (1942)

(*9*) Beisenherz, G., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K.-H., Meyer-Arendt, E., and Pfleiderer, G., Z. *Naturforsch.*, **8b**, 555 (1953)

(*10*) Green, D. E., Herbert, D., and Subrahmanyan, V., *J. Biol. Chem.*, **138**, 327 (1941)

(*11*) Uehara, K., Muramatsu, I., and Kobashi, K., unpublished

(*12*) Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941)

(*13*) Racker, E., *J. Biol. Chem.*, **184**, 313 (1950)

(*14*) Kobayasi, S., *J. Biochem.* (*Japan*), **33**, 301 (1941)

(*15*) Holzer, H., Schultz, G., Villar-Palasi, C., und Jüntgen-Sell, J., *Biochem. Z.*, **327**, 331 (1956)

(*16*) Westenbrink, H. G. K., and van Dorp, D. A., *Nature*, **145**, 465 (1940)

(*17*) Westenbrink, H. G. K., van Dorp, D. A., and Veldman, H., *Enzymologia*, **9**, 73 (1940)

(*18*) Blanchaer, M. C., and Cohen, L. H., *Canad. J. Res. E.*, **27**, 275 (1949)

(*19*) Racker, E., *J. Biol. Chem.*, **167**, 843 (1947)

(*20*) Wetzel, K., *Planta*, **15**, 697 (1931)

(*21*) Holt, C. v., Wiethoff, E. O., Kröner, B., Leppla, W., und Holt, L. v., *Biochem. Z.*, **329**, 119 (1957)

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HYDROGEN INHIBITION AND THE MICHAELIS CONSTANT OF ANAEROBIC NITROGEN FIXATION

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It is well established that molecular hydrogen is a specific and competitive inhibitor of nitrogen fixation by *Azotobacter* and other aerobic fixers (*1*, *2*, *3*). As for the inhibitory effect of hydrogen on anaerobic nitrogen fixation, Rosenblum and Wilson (*4*) reported that the rate of fixation was not appreciably influenced by the presence of molecular hydrogen, although the total amount of fixed nitrogen was only slightly diminished.

Several recent observations suggesting a certain common feature between aerobic and anaerobic nitrogen fixation have been reported. It was found (*5*, *6*, *7*) that nitrogen fixation by *Clostridium* was inhibited by a low concentration of carbon monoxide and by nitrous oxide, both of which had been previously known to be inhibitory for aerobic fixation. Furthermore, observations with *Clostridium* (*8*) indicated a close relationship between hydrogenase and nitrogen fixation, a relation which has long been infered also to exist for *Azotobacter* and certain other bacteria (*9*).

In view of these circumstances, a precise reinvestigation of the effect of hydrogen on the nitrogen fixation by *Clostridium* was undertaken. Since in using our bacterial strain a considerable inhibition by hydrogen was detected, a further attempt was made to determine the effect of various partial pressures of hydrogen and nitrogen on the rate of anaerobic nitrogen fixation.

METHODS

The strain of *Clostridium* (originally isolated from soil)*, procedures for its cultivation, and methods used for nitrogen determination were the same as those employed in the previous report (*7*). Nitrogen-free media containing *M*/20 phosphate buffer and 1 per cent $CaCO_3$ were used throughout the present study.

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* It has been recently found by one of the authors (S. Hino, working in the laboratory of Prof. P. W. Wilson, University of Wisconsin U.S.A., at present) that the bac-

Hydrogen gas was generated from hydrochloric acid and zinc metal and purified by passing through concentrated solution of KOH and saturated solution of $KMnO_4$, alkaline pyrogallol and finally through heated copper granules in succession. Argon gas of more than 99.99 per cent purity was used without further purification*.

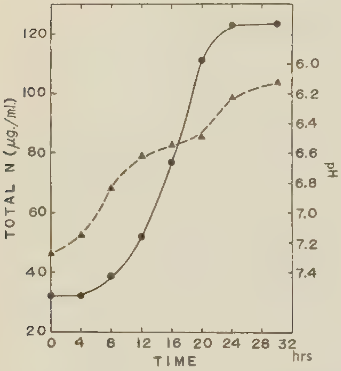

FIG. 1. Time course of nitrogen fixation and pH of medium during the growth of *Clostridium.*

Gas phase was filled with nitrogen to one atmosphere. Culture was shaken at 30°. —●—, total nitrogen (sum of initial and fixed nitrogen) ; --▲--, pH of the medium.

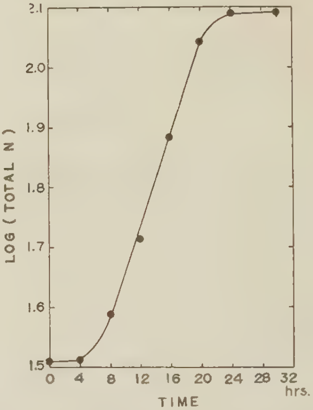

FIG. 2. Relation between log (total N) and culture duration plotted from the results of Fig. 1.

In some of our experiments, particularly those for determination of the Michaelis constant, gas samples were analyzed for accurate composition of the initial gas mixture and for total amount of hydrogen liberated during the growth of culture.

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terium shows marked tolerance for oxygen in glucose nutrient broth. Although many properties of this strain coincide with those of the genus *Clostridium,* the strain would be placed in some other taxonomical positions, *viz.,* in the genus *Aerobacillus,* or the like.

* The authors are grateful for the Tokai Ryuan Kogyo Co. for kindly supplying the argon gas.

RESULTS

Time Course of Nitrogen Fixation—Two or three different flasks filled with 100 per cent N_2 were taken out of the shaker every 4 hours and the amounts of nitrogen and pH were determined for the culture solution in each flask. Results obtained are presented in Figs. 1 and 2. While nitrogen was increasingly fixed, pH of the medium was gradually lowered (Fig. 1) and CO_2 and H_2 were liberated into the gas phase. In spite of the change in pH and the generation of gases (particularly of hydrogen), a fairly good linear relationship was obtained when the logarithm of the total nitrogen* was plotted against time during the culture period from 6 to 20 hours (Fig. 2).

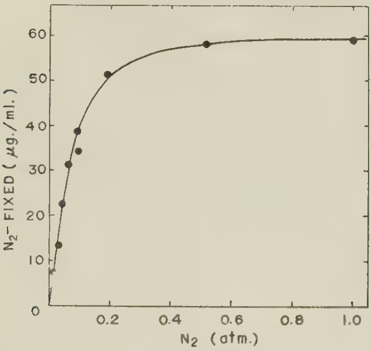

Fig. 3. Effect of partial pressure of nitrogen on nitrogen fixation by *Clostridium*.

Argon was added to the gas phase to make the total pressure up to one atmosphere. Culture was kept shaking for 16 hours at 30°. Initial pH 7.7, initial N 32.2 μg./ml.

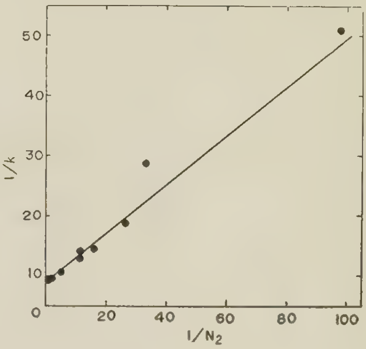

Fig. 4. Evaluation of Michaelis constant for nitrogen fixation by *Clostridium*.

$k = 2.303/10 \log$ (total N at 16 hour/initial N).

Effect of Partial Pressure of Nitrogen—The gas phase was filled with mixtures of various proportion of argon and nitrogen to one atmospheric pressure. In this experiment, the nitrogen content of the culture was analyzed only after 16 hours. As can be seen from Fig. 3, nitrogen fixation remained practically unaffected above 0.2 atmospheres of nitrogen, then it decreased sharply as the partial pressure of nitrogen reached the lower region. Regardless of the difference in growth rate, the pH of the individual culture did not show any significant difference.

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* " Total nitrogen " is the sum of initial (*ca.* 30 μg./ml.) and fixed nitrogen.

Growth rate constant k was calculated by the formula

$$k=2.303/10 \log (\text{final total N}/\text{initial N})$$

on the assumption that the duration of lag was 6 hours*. When the reciprocal of k-value was plotted against the reciprocal of the partial pressure of nitrogen, a straight line was obtained, shown as an example in Fig. 4 which is one out of three experiments. From the slope and point of intercept of line, the Michaelis constant of the nitrogen fixing reaction was estimated to be approximately 0.03 atm. according to the equation of Lineweaver and Burk (*10*).

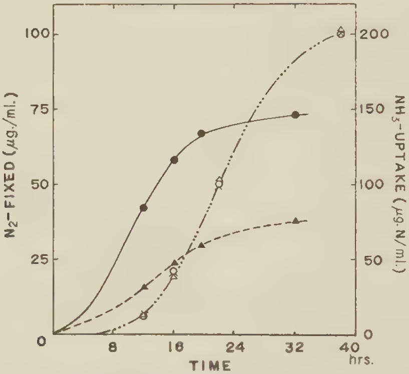

FIG. 5. Effect of hydrogen on both nitrogen fixation and ammonia uptake by *Clostridium*. Initial N 39.0 μg./ml., initial pH 7.7. ○, ● : N_2, 0.2 atm. ; H_2, 0.0 atm. ; Ar, 0.8 atm. △, ▲ : N_2, 0.2 atm. ; H_2, 0.6 atm. ; Ar, 0.2 atm. × : N_2, 0.0 atm. ; H_2, 1.0 atm. ; Ar, 0.0 atm. ——, - - - - - Nitrogen fixation, —···—···— ammonia uptake.

Inhibition by Molecular Hydrogen—According to previous workers (*4*), a slight inhibition of nitrogen fixation was detected in later stage of bacterial growth which led us to investigate the possibility that a distinct inhibition could be obtained when the pH of media was lowered previous to starting the experiment. When we adjusted the initial pH in two preliminary experiments to 6.5 and 5.9, respectively, we found about

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* Even though the actual lag period be changed because of variations in preculture conditions, the final calculation of Michaelis constant cannot be changed regardless of the length of actual lag period.

TABLE I

Effect of Various Partial Pressures of Nitrogen and Hydrogen on Nitrogen Fixation by Clostridium

Experi.	N_2 (*atm.*)	H_2 (*atm.*)	culture time (*hours*)	total N (*μg./ml.*)					growth rate const. k ($k \times 10^4/2.303$)	$\frac{k}{k_i}$
				0	8	12	16	20		
I	0.2	0.0		32.5	37.0	—	—	113.8	406	1.00
		0.2			36.7	—	67.8	92.6	334	1.22
II	0.2	0.0		31.4	43.2	—	92.1	—	410	1.00
		0.4			40.4	51.8	65.7	—	264	1.55
III*	0.2	0.0		35.1	43.3	—	74.8	97.0	389	1.00
		0.6			36.2	41.4	48.5	—	195	1.99
		0.8			—	37.5	42.5	48.1	179	2.17
IV	0.3	0.0		29.4	39.4	62.0	90.6		452	1.00
		0.2			38.7	54.1	76.1		368	1.23
		0.6			35.6	46.2	57.5		261	1.73
V	0.3	0.0		30.7	41.0	60.4	89.7		426	1.00
		0.1			39.7	57.4	79.5		377	1.13
		0.4			37.0	49.3	63.8		296	1.44
VI**	0.4	0.0		34.7	45.8	—	94.1		417	1.00
		0.1			41.7	57.8	83.1		413	1.01
		0.4			43.3	54.6	73.8		319	1.31
VII	0.4	0.0		34.1	47.3	71.2	105.3		434	1.00
		0.2			42.3	59.7	85.6		383	1.13
		0.6			39.5	53.2	71.9		326	1.33
VIII***	0.6	0.0		40.7	51.2	68.2	88.9		399	1.00
		0.2			49.8	65.5	82.2		362	1.10
		0.4			47.3	59.9	77.2		355	1.12
IX	0.8	0.0		31.0	46.7	71.5	107.2		451	1.00
		0.1			44.8	69.6	101.3		443	1.02
		0.2			43.1	65.9	94.3		426	1.06

* Culture hours : 17, 20, 23.5, 26, ** culture hours : 8.75, 12, 16, *** culture hours : 10, 13, 16.

Argon was added to the gas phase to make the total pressure up to one atmosphere. Culture was shaken at 30°. Initial pH 7.7.

38 per cent (pH 6.5) and 44 per cent (pH 5.9) inhibition in the rate of

fixation under the gas mixture of 50 per cent N_2 and 50 per cent H_2. No appreciable inhibition of NH_3-uptake by 100 per cent H_2 was observed.

It was shown by further experiments that the rate of nitrogen fixation is remarkably inhibited by hydrogen even when the initial pH is maintained at 7.7, whereas between 100 per cent argon and 100 per cent H_2 there is no significant difference in the rate of NH_3-uptake (Fig. 5). It can be concluded, at least for our organism, that anaerobic nitrogen fixation is inhibited by molecular hydrogen in a specific manner.

Type of Inhibition by Molecular Hydrogen—Measuring the rate of nitrogen fixation under a mixture of nitrogen and hydrogen in various partial pressures, we sought the answer as to whether the inhibition by hydrogen is competitive or non-competitive. Samples of culture from three or four different flasks in the same condition of gas phase were analyzed at the end of 8, 12, and 16 hours, and the k-values were calculated from the slope of the line obtained by plotting log (total N) against time (Table I). In evaluating the k-values, especially we had to take care of small fluctuation in lengths of growth phases which can cause considerable errors of estimates for the slopes of logarithmic growth curves. The type of inhibition was examined by analyzing the results in the following two equations (*11*),

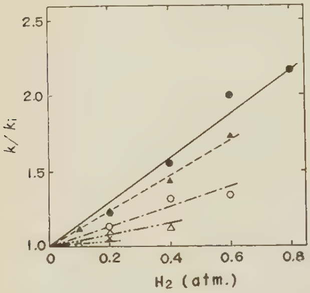

FIG. 6. Competitive inhibition in nitrogen fixation by molecular hydrogen in *Clostridium*.
——●—— : N_2, 0.2 atm. ---▲--- : N_2, 0.3 atm. —·—○—·— : N_2, 0.4 atm. —··△—·· : N_2, 0.6 atm. —···×—··· : N_2, 0.8 atm.

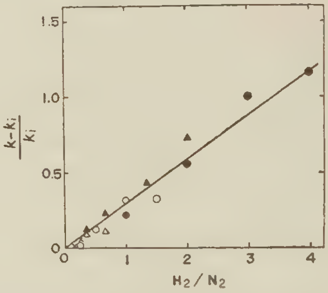

FIG. 7. Evaluation of distribution coefficient between hydrogen and nitrogen in nitrogen fixation by *Clostridium*.
● : N_2, 0.2 atm. ▲ : N_2, 0.3 atm. ○: N_2, 0.4 atm. △ : N_2, 0.6 atm. × : N_2, 0.8 atm.

$$\frac{k}{k_i}=1+\frac{K_s}{K_i}\frac{(I)}{K_s+(S)} \quad \text{......................for competitive inhibition}$$

and $$\frac{k}{k_i}=1+\frac{(I)}{K_i} \quad \text{..............................for non-competitive inhibition}$$

where (S) and (I) are the concentrations of substrate (N_2) and inhibitor (H_2) ; K_s and K_i are, respectively, the Michaelis constant and dissociation constant of enzyme-inhibitor complex ; k and k_i are the velocity in absence and presence, respectively, of inhibitor at a particular substrate concentration.

The slopes of the lines obtained when k/k_i calculated from the data in Table I was plotted against (H_2) are unequivocally dependent on different values of (N_2) as shown in Fig. 6, indicating the inhibition is competitive.

In order to calculate the distribution coefficient K_s/K_i, $(k-k_i)/k_i$ was plotted against $(H_2)/(N_2)$ as shown in Fig. 7. In spite of some inevitable uncertainty in determining k- and k_i-values, the proportionality appeared to hold fairly good, coincidently with the assumption of competitive inhibition. The coefficient was obtained from the slope of the line, and taking the value of K_s as 0.03 atm., the approximate value of K_i was estimated as 0.1 atm.

DISCUSSION

In contrast to the certainty in kinetical studies with purified enzyme preparations, in studies of organisms in growing state some uncertainty is involved in the evaluation of such values as velocity constant or dissociation constant in a particular enzyme reaction. Among the factors which might affect the evaluation of constants in the present study, metabolic hydrogen produced from carbohydrate may have some importance. In our culture condition, about 0.1 atm. of metabolic hydrogen was accumulated in the gas phase during a bacterial growth of about 16 hours when the partial pressure of nitrogen was between 0.2 and 1.0 atm. It is likely that the inhibition by this metabolic hydrogen is significant when the partial pressure of nitrogen is low. The net result could be such that the obtained value of the Michaelis constant would be a little higher than the true value. This effect of hydrogen, along with some other inaccuracies in evaluating the k-value of the fixation, gives only an approximate value of K_{H_2}.

The Michaelis constant and the K_{H_2} values for aerobic nitrogen fixation have been estimated by the elaborate works of Wilson and his colleagues. The reported values of K_{N_2} are 0.02 atm. for *Azotobacter* (*12*), 0.05 atm. for symbiotic system (*1*), and 0.02 atm. for *Nostoc* (*3*). The nature of observed hydrogen inhibition is competitive and the values of K_{H_2} are 0.11 atm. for *Azotobacter* (*2*), and 0.15 atm. for symbiotic system (*1*). Even taking into consideration that there is some uncertainty in our estiamation, the observed values of 0.03 atm. as the Michaelis constant and 0.1 atm. as K_{H_2} in the present study closely approximate those obtained for the aerobic fixation. These calculated results and the competitive nature of hydrogen inhibition, together with the reported inhibition by carbon monoxide and nitrous oxide (*5, 6, 7*), indicate a striking similarity between the mechanism of aerobic and anaerobic fixation.

The situation, however, still remains somewhat complicated in view of the study reported by Rosenblum and Wilson (*4*). With *Clostridium pasteurianum* W5 as a test organism and under an atmosphere of 0.2 atm. N_2 plus 0.6 atm. H_2, they could detect no significant inhibition in the rate of nitrogen fixation and only 10–20 per cent decrease in the total amount of nitrogen. These results differ remarkably from our observations obtained with a similar gas mixture as is apparent from the results presented in Fig. 5. Further investigation is needed to reveal the cause of these apparently contradictory experimental results.

SUMMARY

1. Using a strain of *Clostridium* originally isolated from soil, the effect of partial pressure of nitrogen as substrate and hydrogen as inhibitor on the process of anaerobic nitrogen fixation was studied.

2. The rate of nitrogen fixation decreased in the presence of molecular hydrogen and the inhibition was found to be of competitive nature. No inhibition of ammonia-uptake by hydrogen was observed.

3. The Michaelis constant of the anaerobic nitrogen fixation was estimated to be about 0.03 atmosphere and the apparent dissociation constant of hydrogen-enzyme complex was found to be approximately 0.1 atmosphere. Both values are almost the same as those obtained by previous workers in aerobic nitrogen fixation.

For gas analysis in this work thanks are due to Assist. Prof. T. Koyama and Miss H. Tanaka of the Chemical Institute, Faculty of Science, Nagoya University. This work is supported in part by Grant in Aid for Scientific Research from the Ministry of Education.

REFERENCES

(*1*) Wilson, P. W., The biochemistry of symbiotic nitrogen fixation, University of Wisconsin Press, Madison (1940)

(*2*) Wyss, O., Lind, C. J., Wilson, J. B., and Wilson, P. W., *Biochem. J.*, **35**, 845 (1941)

(*3*) Burris, R. H., and Wilson, P. W., *Botan. Gaz.*, **108**, 254 (1946)

(*4*) Rosenblum, E. D., and Wilson, P. W., *J. Bacteriol.*, **59**, 83 (1950)

(*5*) Virtanen, A. I., Mustakallio, H., and Strandström, H., *Soumen Kemistilehiti*, **26 B**, No. 2, 6 (1953)

(*6*) Virtanen, A. I., and Lundbom, S., *Acta Chem. Scand.*, **7**, 1223 (1953)

(*7*) Hino, S., *J. Biochem.*, **42**, 775 (1955)

(*8*) Shug, A. L., Hamilton, P. B., and Wilson, P. W., Inorganic nitrogen metabolism, p. 344, Johns Hopkins Press, Baltimore (1956)

(*9*) Wilson, P. W., Bacterial physiology, p. 467, Acad. Press, New York (1951)

(*10*) Lineweaver, H., and Burk, D., *J. Amer. Chem. Soc.*, **56**, 658 (1934)

(*11*) Wilson, P. W., Respiratory enzymes, p. 16, Revised ed., Burgess Publishing Co., Minneapolis (1949)

(*12*) Wilson, P. W., Burris, R. H., and Lind, C. J., *Proc. Natl. Acad. Sci. U.S.*, **28**, 243 (1942)

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ON THE FORMATION OF GENTISIC ACID. II

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Gentisic acid was recently found to be formed from homogentisic acid by the liver extract of rabbit in the presence of nitroso-R salt (*1*). Furthermore this compound was excreted in the urine of the rabbit receiving tyrosine orally, when nitroso-R salt was injected subcutaneously at the same time (*1*). Since ferrous ion is indispensable for the enzymatic degradation of homogentisic acid to fumarylacetoacetic acid (*2*), these facts suggest that nitroso-R salt would remove ferrous ion, resulting in the shift of the normal pathway of homogentisic acid.

In this paper data on the excretion of homogentisic acid in the urine of a hereditary and experimental alcaptonuria are presented. For this purpose a differential determination of gentisic acid and homogentisic acid was accomplished.

EXPERIMENTALS AND RESULTS

I. Estimation of Gentisic Acid in the Urine

The urines of guinea pig were collected for 24 hours in bottles which contained 2 ml. of 20 per cent H_2SO_4 to keep the urines acid and filtered. The filtrate was extracted with ether and the etherial layer was evaporated to a volume of about 20 ml. To the concentrated ether extract 10 ml. of $M/2$ phosphate buffer (pH 7.2) was added and shaken. The layer of phosphate buffer was acidified by the addition of 1 ml. of 20 per cent H_2SO_4 and extracted with butylacetate saturated with water. Then the butylacetate extract was used for the fluorometric estimation (Photovolt) of gentisic acid. 50 μg of gentisic acid was dissolved in 20 ml. of butylacetate saturated with water and its ultraviolet absorption curve was taken. Since gentisic acid has the maximal absorption at 340 mμ, B-Hg-1 (360 mμ) was used as the primary filter (Fig. 1) and from the data shown in Table I, B-470 was used as the secondary filter for the fluorometry (Photovolt).

Recovery of Gentisic Acid—Various amounts of gentisic acid dissolved in 20 ml. of normal urine or distilled water were treated as described above. The both butylacetate extracts were estimated fluorometrically and the values obtained were compared with those from the solutions containing the same amounts of gentisic acid, dissolved in 20 ml. of butylacetate saturated with water. As shown in Fig. 2, 92 per cent of the added gentisic acid was recovered.

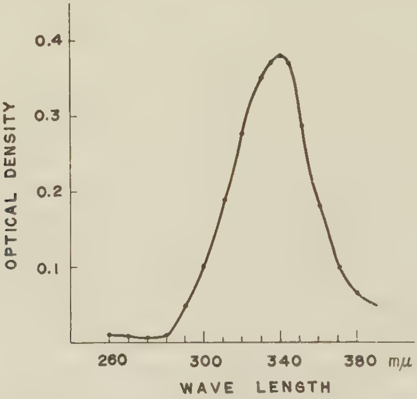

FIG. 1. Ultraviolet absorption spectra of gentisic acid (12 μg. per ml.) in butylacetate saturated with water.

TABLE I

Interrelation between Filter of Fluorometer and Degree of Fluorescence of Gentisic Acid

Filter	Waue length mμ	Degree of fluorescence
N-440	410–480	3000
B-470	410–580	5020
M-490	480–515	880

50 μg. of gentisic acid in 20 ml. of butylacetate, saturated with water. B-Hg-1 as primary filter.

Determination of Gentisic Acid and Homogentisic Acid—Since homogentisic acid has no fluorescence, gentisic acid can be easily differentiated fluorometrically from homogentisic acid. On the contrary very little gentisic acid is formed under the following conditions as compared with homogentisic acid. Therefore, though both compounds give positive Brigg's reaction (*3*), the values obtained by this method (*3*) can be regarded as these of homogentisic acid.

II. Excretion of Gentisic Acid in the Urine of a Hereditary and Experimental Alcaptonuria

A Hereditary Alcaptonuria—Urine of an alcaptonuria, who excretes more than 7 g. of homogentisic acid every day, was collected. Kapp *et al.* (*4*) have demonstrated that salicylic acid was converted to gentisic acid, consequently the administration of salicylic acid preparations were prohibited strictly. The urine during 24 hours was extracted with a same volume of ether and the ether was evaporated on a water bath to a sirup. The residue was dissolved in 5 ml. of distilled water, and then saturated lead acetate solution was added until no more precipitate was formed. The mixture was kept in a refrigerator overnight, then the precipitate was gathered and dissolved in a small amount of water. 25 per cent H_2SO_4 was added to the solution until no more white precipitate appeared. The formed precipitate was removed, and the supernatant fluid was extracted with the same volume of ether. The ether was

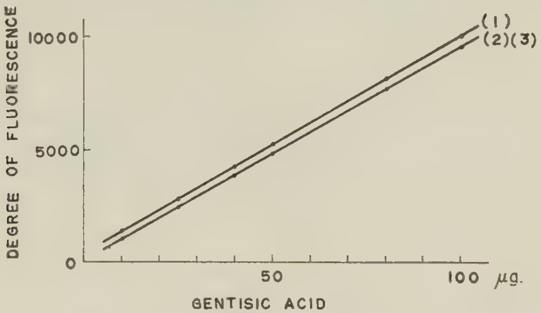

FIG. 2. Recovery ratio of gentisic acid.

(1) Gentistic acid dissolved in 20 ml. of butylacetate.

(2) Gentisic acid was added to 20 ml. of urine, then extracted with 20 ml. of butylacetate.

(3) Gentisic acid was added to 20 ml. of distilled water, then extracted with 20 ml. of butylacetate.

evaporated and the resulting sirup was dissolved in 0.2 ml. of water and subjected to the paperchromatography with the following two kinds of solvent systems; isopropanol-ammonia-water (8:1:1) system and butanol-acetic acid-water (4:1:5) system. Gentisic acid was detected by its fluorescence and its reduction by ammonia-alkaline $AgNO_3$, homogentisic acid by its reduction reaction.

As shown in Fig. 3, gentisic acid was demonstrated to be excreted in the urine of the hereditary alcaptonuria. These experiments were repeated five times and the occurrence of gentisic acid was confirmed in all samples without exception.

The spot corresponding to gentisic acid on the paperchromatogram was cut off and eluted from paper with butylacetate. Gentisic acid was confirmed from its characteristic spectrum, which has the absorption maximum at 340 mμ.

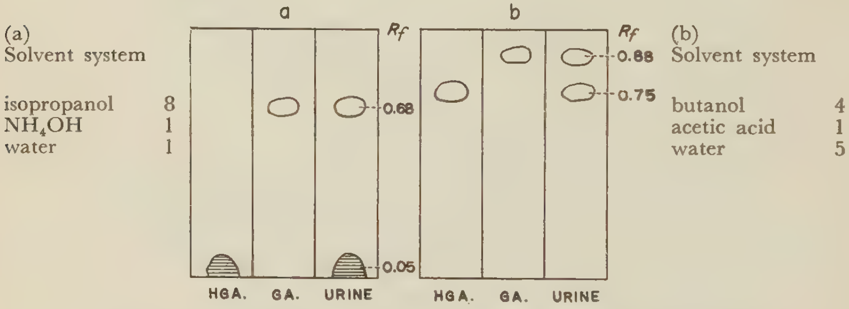

FIG. 3. Ascending paperchromatogram of the urine of the alcaptonuric patient. HGA.: homogentisic acid, GA.: gentisic acid.

Gentisic acid in the urine of the alcaptonuria was determined by the method described above and shown to be excreted by 0.5–0.6 mg. in a day. When 2 g. of tyrosine was administered orally to the alcaptonuria, the excretion of gentisic acid increased a little but in the urine of normal individual neither gentisic acid nor homogentisic acid was detected after the same treatment.

Experimental Alcaptonuria—Guinea pigs, weighing 200–250 g., were fed on a following diet (Table II) supplemented with fresh vegetables for few weeks and the animals growing normally (300–350 g.) were selected to use for the experiment.

The urine was collected in a bottle, to which 2 ml. of 20 per cent

H_2SO_4 was added previously and homogentisic acid and gentisic acid excreted in the urine during 24 hours were determined daily as described above.

As shown in Fig. 4, the excretion of homogentisic acid commenced after 8 days on a scorbutic diet, and reached a maximum value on the 13th day, then decreased day by day until no more excretion occurred on the 20th day.

Table II

Composition of a Scorbutic Diet

Okara (insoluble soyabean residue)	90 per cent
Crusta of wheat	5
Casein	3
Butter	2

The mixture was heated for 1 hour at 100°, then a small volume of water was added.

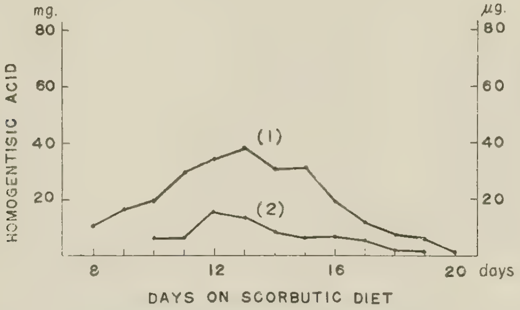

Fig. 4. Excretion of homogentisisc acid (1) and gentisic acid (2) in an ascorbic acid deficient guinea pig.

Gentisic acid also appeared, and its excretion reached a maximum a few days later than that of homogentisic acid, thereafter it disappeared one day earlier than homogentisic acid (Fig. 4).

When tyrosine (oral) and homogentisic acid (subcutaneous) were given to the scorbutic guinea pigs respectively, the excretions of homogentisic acid and gentisic acid increased and these excretions were depressed by the administration of vitamin C (2 mg. subcutaneous) (Figs. 5 and 6).

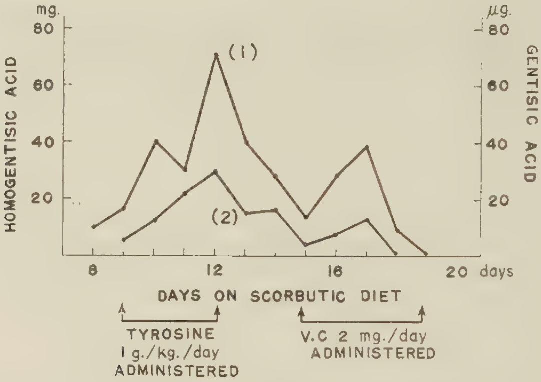

Fig. 5. The influence of administration of tyrosine or ascorbic acid on the excretion of homogentisic acid (1) and gentisic acid (2) in a scorbutic guinea pig.

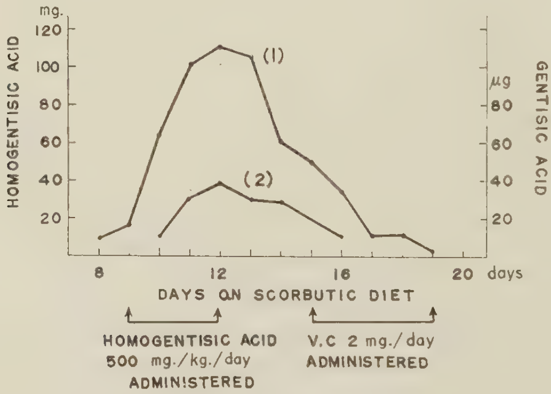

Fig. 6. The influence of administration of homogentisic acid on the excretion of homogentisic acid (1) and gentisic acid (2) in an ascorbic acid deficient guinea pig.

Using paperchromatography both acids were detected in the urine 13–15 days after being fed on a scorbutic diet, and keeping the animals on the same diet for longer depressed the excretion of these compounds, resulting that of only *p*-hydroxyphenylpyruvate. At this time the injection of 2 mg. of vitamin C gave rise to the excretion of both acids next day, and the continuance of this treatment resulted the disappearance of these compounds in the urine. These facts seem to suggest that homogentisic acid was degraded completely through its ordinary pathway in the normal animal with sufficient ascorbic acid, and that even the formation of homogentisic acid from *p*-hydroxyphenylpyruvate did not occur in animals deprived of this vitamin.

Enzymatic Experiment—A guinea pig on a scorbutic diet for 11–13 days was sacrificed by bleeding and the liver was ground with same amount of sea sand. The paste was extracted with two volumes of *M*/10 phosphate buffer (pH 7.2) in the refrigerator for an hour and centrifuged. The supernatant was used for the enzymatic experiments. O_2-uptake was measured in the Warburg's manometer, and acetoacetic acid formed was determined by the method using aniline citrate (*5*). Gentisic acid was measured as described above. The conditions of enzymatic reaction were as follows ;

TABLE III

Degradation of Homogentisic Acid or Gentisic Acid by Liver Extract of Normal or an Ascorbic Acid Deficient Guinea Pig

	Substrate	Addition	Oxygen uptake (atom per μM of HGA)	Acetoacetate formed (mole per μM of HGA)
Liver extract of the normal guinea pig	HGA 2 μM	no addition	1.9	0.9
		V.C. 1 mg.	1.9	0.9
		GA 2 μM	1.9	0.9
	GA 2 μM		0.0	0.0
Liver extract of an ascorbic acid deficient guinea pig	HGA 2 μM	no addition	1.15	0.6
		V.C. 1 mg.	1.85	0.8
		GA 2 μM	1.15	0.6
	GA 2 μM		0.0	0.0

Condition : 37.5°, 90 minutes. HGA : homogentisic acid
GA : gentisic acid

Main chamber:	1 *M* phosphate buffer (pH 7.4)	0.3 ml.
	enzyme solution	1.0

	added distilled water	1.2
Side arm:	homogentisic acid (2 μM)	0.5
Center well:	50 per cent KOH	0.1
	37.5°, 90 minutes	

The incubated solution was deproteinized by the addition of 1 ml. of 30 per cent trichloroacetic acid and extracted with 20 ml. of ether. The results are shown in Table III.

From the data of Table III, it was shown that the liver extract of a scorbutic guinea pig has lower activity to degrade homogentisic acid to acetoacetic acid than that of the normal, and the addition of vitamin C restored the activity considerably. Gentisic acid was not degraded and showed no effect on the activity of both liver extracts.

After 10 ml. of liver extract was incubated in the presence of 5 mg. of homogentisic acid at 37.5° for 3 hours, the mixture was deproteinized and extracted with two volumes of ether. The ether extract was evaporated and the residue was dissolved in about 0.2 ml. of water, which was used for the paperchromatography. The presence of gentisic acid was demonstrated.

SUMMARY

1. Gentisic acid was confirmed to be eliminated in the urine of hereditary and experimental alcaptonuria by the paperchromatography.

2. A method of differential determination of homogentisic acid and gentisic acid in the urine was accomplished. Gentisic acid was observed to be excreted in the urine of an alcaptonuria, who eliminates *ca.* 7 g. of the latter daily and to be increased slightly by the administration of tyrosine (2 g.) to him.

3. Gentisic acid was demonstrated to appear later and to disappear earlier than homogentisic acid in the urine of prolonged ascorbic acid deficient guinea pig. Both acids increased after the administration of tyrosine and disappeared after that of ascorbic acid in the urines of the scorbutic guinea pig.

4. The formation of gentisic acid from homogentisic acid by liver extract of scorbutic guinea pig was demonstrated by means of paperchromatography. This acid was not oxidized further by the liver extract and had not any effect on the enzymatic degradation of homogentisic acid.

REFERENCES

(*1*) Ichihara, K., Ikeda, S., and Sakamoto, Y., *J. Biochem. (Japan)*, **43**, 129 (1956)
(*2*) Suda, M., and Takeda, Y., *J. Biochem. (Japan)*, **37**, 381 (1950)
(*3*) Briggs, A. P., *J. Biol. Chem.*, **51**, 453 (1922)
(*4*) Kapp, M., and Coburn, F., *J. Biol. Chem.*, **145**, 549 (1942)
(*5*) Edson, N. L., *Biochem. J.*, **29**, 2082 (1935)

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STUDIES ON POLYOL DEHYDROGENASE FROM MYCOBACTERIUM AVIUM

In 1898, the selective oxidation of polyols by *Acetobacter* was discovered by Bertrand (*1*), whose observation was generalized in a rule that was extended by Hudson *et al.* (*2*). Recently, Edson and his collaborators (*3–6*) reported the specificity of the polyol dehydrogenases of different organisms, and described that there are at least three types of polyol dehydrogenases: L-iditol, D-mannitol, and galactitol dehydrogenases.

This communication deals with the specificity and multiplicity of polyol dehydrogenase in *Mycobacterium avium* and it will be shown that, by cell-free extracts, myo-inositol, D-mannitol, glycerol, propanediol-1, 2, and ethylene glycol are oxidized.

Mycobacterium avium (strain Takeo) was grown in Sauton medium for 7 days. The washed cells were quickly desiccated *in vacuo* over P_2O_5. A portion of these dried cells was finely ground by 4 portions of quartz sand, and subsequently extracted with 30 volumes of 0.1 N Na_2HPO_4. After keeping overnight in an icebox, the cells were centrifuged off at 3,000 r.p.m. for 30 minutes and the supernatant fluid was recentrifuged at 10,000 r.p.m. for 20 minutes at 0°. The supernatant fluid was dialyzed overnight against running-water. To remove nucleotides, 1 per cent protamine sulfate was added to the dialyzate. After centrifugation, the supernatant fluid was used as enzyme solution. In some experiments, the fraction precipitated by ammonium sulfate (between 20 to 70 per cent saturation) was also used. The enzyme activities are retained for several weeks if the enzyme preparation is frozen.

The enzyme assay is carried out by the measurement of the rate of reduction of triphenyltetrazolium chloride (TTC) at 500 mμ in the presence of methylene blue or of 2,6-dichlorophenolindophenol (DCPP) at 600 mμ (*7*). As the crude enzyme preparation contains DPNH-cytochrome c reductase, the election carrier system for dyes is complete in the enzyme assay system.

When the activity of the enzyme towards polyols was measured by the rate of TTC-reduction, myo-inositol, D-mannitol, glycerol, propanediol-1, 2, and ethyleneglycol were found to be oxidized, while D-sorbitol, D-dulcitol, and erythritol were resistant.

Fig. 1 indicates the requirement of diphosphopyridine nucleotide (DPN) for the oxidation of myo-inositol and D-mannitol: in the presence of DPN, DCPP was reduced rapidly; however in the absence of DPN, the dye was reduced slowly. The rates of oxidation of myo-inositol and D-mannitol were determined by spectrophotometric measurement of reduced DPN at 340 mμ (Fig. 2). As can be seen from the result that D-fructose oxidizes reduced DPN in the presence of the enzyme, hence the oxidation of D-mannitol is reversible.

As shown in Table I, inhibitors and metal-chelating agents were tested for their ability to affect dehydrogenase activity.

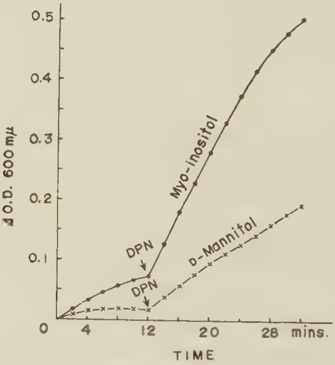

FIG. 1. Requirement of DPN for the oxidation of myo-inositol and D-mannitol.

Reaction mixture contains: enzyme (protamine supernatant), 1400 μg.; DCPP, 60 μg.; myo-inositol or D-mannitol, 300 μM; DPN, 0.032 μM; Na_2HPO_4, 30 μM. Total volume, 3.0 ml. at 20°.

Table II indicates that each of Mg, Mn, and Co ions activated about 3-fold the oxidation of D-mannitol, but only slightly that of myo-inositol.

These results strongly suggest that D-mannitol dehydrogenase is a DPN-linked, metal-containing, sulfhydryl enzyme. From the evidence that D-mannitol dehydrogenase does not attack D-sorbitol, *Mycobacterium avium* seems to be more limited than *Acetobacter suboxydans* with respect to the substrate specificity of D-mannitol dehydrogenase. Moreover, as Dr. Edson kindly suggested in his private communication (*8*), if the enzyme required DPN which is readily dissociated from the apoenzyme, it is quite possible that the stereospecificity will be different from the specificities of *Acetobacter suboxydans*. Recently, the multiplicity of polyol dehydrogenases was also indicated by the studies of xylitol dehydrogenase from pig-liver (*9*) and sorbitol dehydrogenase from *Acetobacter suboxydans* (*10, 11*).

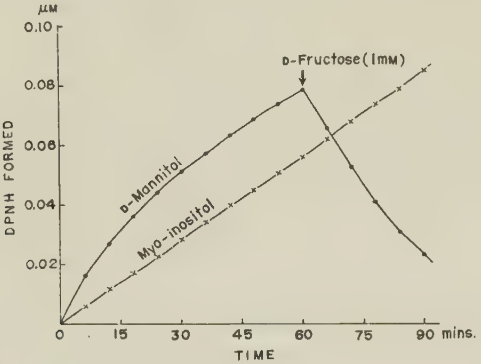

FIG. 2. Reduction of DPN.

Reaction mixture contains : enzyme (protamine supernatant) 1.0 ml. ; myo-inositol or D-mannitol, 500 μM ; DPN, 0.32 μM ; Na_2HPO_4, 43 μM. Total volume 3.0 ml. at 20°.

TABLE I

Inhibition

Inhibitor		myo-Inositol	D-Mannitol
		(%)	(%)
EDTA,	5×10^{-3} *M*	81	85
Nitrose-R,	1×10^{-3} *M*	—	100
PCMB,	2.5×10^{-5} *M*	100	100
Hg^{++}	5×10^{-4} *M*	100	—
NaF	1×10^{-2} *M*	100	91

Reaction mixture contains : enzyme (ammonium sulfate precipitated fraction) 1500 μg. ; TTC, 0.3 μM ; methylene blue, 0.1 μM ; DPN, 100 μg. ; myo-inositol or D-mannitol, 100 μM ; Na_2HPO_4, 20 μM ; inhibitors as indicated. Total volume 1.0 ml. at 38°, 150 minutes.

TABLE II

Participation of Metal Ions for D-*Mannitol Dehydrogenase*

Metal		Formazan formed (μM)	
		myo-Inositol	D-Mannitol
None		0.12	0.06
Mn^{++}	1.3×10^{-3} *M*	0.15	0.19
Co^{++}	1.3×10^{-3} *M*	0.15	0.20
Mg^{++}	1.3×10^{-3} *M*	—	0.18
Zn^{++}	1.3×10^{-3} *M*	0.17	0.07

Reaction mixture contains: enzyme (the fraction which is precipitated by ammonium sulfate, then dialyzed against EDTA solution), 1510 μg.; TTC, 1.0 μM; methylene blue, 0.5 μM; DPN, 50 μg.; myo-inositol, 300 μM or D-mannitol, 200 μM; Na_2HPO_4, 20 μM; metal ions as indicated. Total volume 1.0 ml. at 38°, 120 minutes.

REFERENCES

(*1*) Bertrand, G., *C. R. Acad. Sci., Paris*, **126**, 762 (1898)
(*2*) Richtmeyer, N. K., Stewart, L. C., and Hudson, C. S., *J. Am. Chem. Soc.*, **72**, 4934 (1950)
(*3*) McCorkindale, J, and Edson, N. L., *Biochem. J.*, **57**, 518 (1954)
(*4*) Arcus, A. C., and Edson, N. L., *Biochem. J.*, **64**, 385 (1956)
(*5*) Shaw, D. R. D. *Biochem. J.*, **64**, 394 (1956)
(*6*) Edson, N. L., Report of the Australian and New Zealand Association for the Advancement of Science, 29th meeting, Sydney, August, p. 281 (1952)
(*7*) Mahler, H. R., Methods in Enzymology, p. 553, Academic Press Inc., Publishers, New York (1955)
(*8*) Edson, N. L., Private communication (1957)
(*9*) Hollmann, S., and Touster, O., *J. Biol. Cpem.*, **225**, 87 (1957)
(*10*) Commins, J. T., King, T. E., and Cheldelin, V. H., *J. Biol. Chem.*, **224**, 323 (1957)
(*11*) Commins, J. T., Cheldelin, V. H., and King, T. E., *J. Biol. Chem.*, **226**, 301 (1957)

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ENZYME FORMATION IN LYSOZYME LYSATE OF BACILUS SUBTILIS

It was already reported that the whole lysate obtained by the treatment of *Bacillus subtilis* cells with lysozyme can still form amylase if boiled extract of cells cultivated in the phase of active amylase production is added (*1*, *2*, *3*). The factor discovered in this extract was studied and established to be of ribonucleic acid (RNA) nature (*2*, *4*). However, in the earlier investigation, the conditions for obtaining constantly active lysate was not so easy to set up. The problems were examined and now it was achieved to devise an adequate procedure for it.

The RNA and protein contents of preparation were found to decrease markedly during the treatment with lysozyme or subsequent incubation period. The previous demonstration of the necessity of active factor (RNA) for the formation of amylase was now interpreted based on the diminished content of RNA in the preparation. Both polyvinyl sulfate (PVS), which was supposed to inhibit ribonuclease (RNAase), and purified protease inhibitor of potato extract (*5*), which was shown to inhibit the action of cyrstalline protease of this bacterium, were added to the incubation mixture of lysozyme treatment. Apparently PVS did not inhibit the lysozyme action as judged from the measurement of optical density (O.D.) decrease, and yet, the ability of amylase formation of the resultant preparation was remarkably high, almost of the comparable order to that of intact cells, while the lysate prepared in the absence of PVS had only a weak activity (Table I).

As already reported (*1*, *3*), the protoplast prepared from cells which are producing amylase actively is unstable and the preparation obtained does not contain intact protoplast. The active preparation could be obtained without any regulation of osmotic tonicity (Table I). Fractionation of whole lysate showed that the major activity remained with the cell residue (precipitate fraction obtained after the centrifugation at 900 g. for 15 minutes) as in the previous studies (*3*). This residual preparation is fairly transparent under the phase contrast microscope, confirming the greater loss of cellular materials as indicated by O.D. measurement or chemical analysis. It is completely gram-negative, but still stained with Alcian Blue 8GN and its shape is somewhat damaged or distorted, indicating partially digested and ruptured cell wall to which are still attached some remaining cellular components. Electron microscope examination also confirmed the above observation (Fig. 1).

The preparation retained the ability of glucose oxidation and of C^{14}-glycine incorporation into protein and nucleic acid at rates comparable to those of intact cells (Table II).

parable to those of intact cells (Table II).

The respiration, amino acid incorporation and amylase formation of the preparation proceeded linearly at least for 4 hours. In addition to amylase, the formation of protease was also demonstrated in this preparation in the absence of protease inhibitor (the increase from 8 units to 28 units per ml. during 3.5 hours in one experiment). The preparation is amenable to RNAase treatment in contrast to intact dcells. RNAase treatment inhibited amylase formation, but not glucose oxidation (Table II).

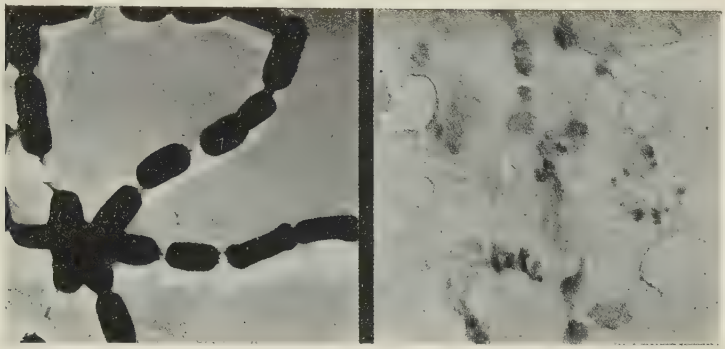
TABLE I

The Effect of Polyvinyl Sulfate (PVS) during the Lysozyme Treatment on the Amylase Forming Ability of the Resultant Preapration

Washed cells of *B. subtilis* N (25 mg. dry weight/ml.) were treated with lysozyme (18 μg./ml.) in the presence of PVS (1.5 mg./ml.) and lactose (5%) as indicated in the table. Phosphate (0.05 *M*, pH=7.3), $MgSO_4$ (0.003 *M*), purified protease inhibitor of potato extract and the crude boiled cell extract were added to all the flask. Lysis was followed by measuring the O.D. of the solution at 570 mμ. After 1 hour at 30° the lysis was complete (initial O.D.) and samples were taken for the measurement of initial amount of amylase (assayed according to the method of Hagihara (*7*). Incubation was continued for 3.5 hours at 30° under aeration.

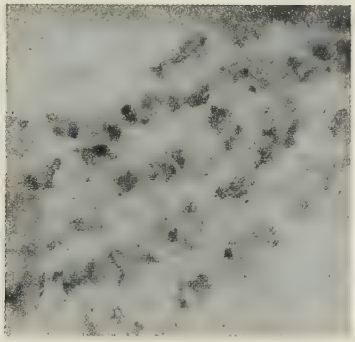
Conditions for lysozyme treatment			Initial O.D.		Amylase ($D^{30'}_{40}$/*ml.*)			
Lysozyme	PVS	Lactose		% of control	Initial	Final	Increase	% of control
−	+	+	0.488	100	3.2	8.3	5.1	100
+	−	+	0.098	20	3.2	3.7	0.5	9.8
	+	+	0.117	24	3.3	6.4	3.1	61
	+	−	0.165	34	3.8	9.0	5.2	102

Though protease inhibitor was shown to prevent effectively the decrease of protein content of the preparation, the experimental results indicated that, in contrast to PVS, it is not necessary for the preparation of lysate which is active in the amylase formation. A number of active lysate obtained in the presence of PVS was analysed. Usually, the ratio of nucleic acid to protein was found to be greater than that of the preparation obtained without added PVS, suggesting the inhibition of intracellular RNAase as expected at first. However, the results are not yet conclusive and the role played by PVS must still await the future investigation.



(a) Control intact cells in PVS and lactose.

(b) Treated with lysozyme in lactose



(c) Treated with lysozyme in PVS and lactose. Preparations were those described in Table I.

Fig. 1. Electronmicrographs of various preparations of *Bacillus Subtilis* N, ×5,000.

Recently, Spiegelman demonstrated the enzyme formation and nucleic acid synthesis in the osmotically shocked protoplast (*6*). Our

TABLE II

The Effect of the Presence of PVS during the Lysozyme Treatment on tpe Ability of Amylase Formation, C^{14}-glycine Incorporation and Respiration of the Resultant Preparation

Washed cells (45 mg. dry weight/ml.) were treated with lysozyme (18 μg./ml.) in the presence of lactose (5%), phosphate (0.05 *M*, pH 7.3), $MgSO_4$ (0.003 *M*) and purified protease inhibitor of potato extract. PVS (1.5 mg./ml.) and RNase (500 μg./ml.) were added as indicated in the table during the course of this treatment. The treatment was continued for 95 minutes in experiment 1 and 75 minutes in experiment 2 at 30°. Samples were taken for the measurement of glucose oxidation (in experiment 1), the chemical analysis, and the assay of initial amount of amylase. In experiment 1, the equal volumes of the crude factor solution (boiled extract of cells containing lactose (5%), phosphate (0.05 *M*, pH=7.3) and $MgSO_4$ (0.003 *M*)) were added. In experiment 2, the equal volumes of C^{14}-glycine-amino acids solution (2-C^{14}-glycine, 400 μg./ml., specific activity=394,000 c.p.m./mg., and other 18 non-labeled amino acids, each 400 μg. L-form/ml.) were added. Incubations were performed for 3.5 hours. For the chemical analysis and isotope measurement, samples were fractionated according to the method of Schneider (*8*). Nucleic acid (NA) was determined by the O.D. measurement at 260 mμ and protein was determined by the method of Lowry *et al.* (*9*). Both values were expressed as per cent of control values (the values in the intact cells in the presence of PVS). Qo_2 for glucose was calculated by subtracting the values for autorespiration.

	Conditions for lysozyme treatment			Chemical analysis (% of control)			Amylase ($D^{35'}_{400}$/*ml.*)				Glucose oxidation		C^{14}-glycine incoroporation			
													NA		Protein	
Extl. No.	Lysozyme	PVS	RNAase	Protein	NA	NA/Protein	Initial	Final	Increase	% of control	Qo_2*	% of control	c.p.m./*ml.*	% of control	c.p.m./*ml.*	% of control
1	—	—	—	101	103	1.02	1.9	9.8	7.9	125	14.4	97				
		+	—	100	100	1.00	1.8	8.1	6.3	100	14.8	100				
		+	+	100	99	0.99	1.8	11.5	9.7	154	—	—				
	+	—	—	22	23	1.04	1.9	2.2	0.3	4.8	3.0	21				
		+	—	41	46	1.12	2.0	4.9	2.9	46	11.4	77				
		+	+	26	19	0.73	1.8	3.6	1.8	29	12.3	83				
2	—	+	—	100	100	1.00	4.0	8.7	4.7	100			904	100	2409	100
	+	+	—	58	87	1.50	3.6	7.7	4.1	87			1390	154	2120	88

* μl of O_2 consumed per hour per preparation derived from 1 mg. dry weight of original cells.

preparation seems to show the similar characteristics on several points, though the method of preparation and hence its nature is quite different in principle. The studies of amylase or any other enzyme formation in our preparation as a subcellular system seem to be promising.

The author wish to thanks to Prof. S. Akabori for his interest and helpful advice in this investigation and also to Dr. M. Ishimoto for his kind supply of polyvinyl sulfate.

REFERENCES

(*1*) Nomura, M., Maruo, B., and Akabori, S., *J. Biochem.*, **43**, 251 (1956)
(*2*) Nomura, M., *J. Biochem.*, **44**, 87 (1957)
(*3*) Nomura, M., in Symposia for Society of Cellular Chemistry, 6, Maruzen, Tokyo (1957)
(*4*) Nomura, M., Hosoda, J., and Yoshikawa, H., to be published in *J. Biochem.*
(*5*) Matsushima, K., *J. Agr. Chem. Soc. Japan*, **29**, 883 (1955) ; **31**, 38 (1957)
(*6*) Spiegelman, S., in the Chemical Basis of Heredity, Johns Hopkins Univ. Press, Baltimore (1957)
(*7*) Hagihara, B., *Ann. Rep. Scient. Works, Fac. Sci. Osaka Univ.*, **2**, 35 (1954)
(*8*) Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945)
(*9*) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951)

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